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RIFT VALLEY FEVER; THE NEUROTROPIC ADAPTATION OF THE VIRUS AND THE EXPERIMENTAL USE OF THIS MODIFIED VIRUS AS A VACCINE.

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In 1944 Rift Valley fever virus was isolated from 6 different lots of mosquitoes captured in uninhabited forest in western Uganda during investigations, the objective of which was to determine the forest vector of yellow fever (Smithburn, Haddow and Gillett, 1948). In the hope of demonstrating the presence of yellow fever virus in wild-caught mosquitoes, suspensions of the insects were inoculated intracerebrally into mice. The 6 strains isolated were not immediately recognized as Rift Valley fever virus. Although histological studies in a second passage mouse gave a clue to the identity, and although it was found that the agents were pathogenic by extraneural inoculation, these 6 and 2 other strains recovered from persons infected in the laboratory were transmitted by intracerebral passage, simply because of technical facility. All the strains so passed in series ultimately lost their hepatotropism and retained their neurotropic properties. Controlled experiments were then undertaken to determine whether this modification of the virus could be brought about regularly by intracerebral passage. When this was found to be the case, studies were made to determine the efficacy of the modified (neurotropic) virus as an immunizing agent. The results of these investigations are the subject of the present communication.

Mackenzie and Findlay (1936) and Mackenzie, Findlay and Stern (1936) found that the neurotropic properties of Rift Valley fever virus could be retained or enhanced, with simultaneous diminution of the hepatotropism, by serial intracerebral passage of the virus in mice previously injected intraperitoneally with specific Rift Valley fever immune serum. The experiments here reported indicate that the use of the immune serum is not necessary, although it may expedite the modification of the virus.

MATERIALS AND METHODS.

All of the strains of virus used in this investigation and most of the methods employed in studying them have been described elsewhere (Smithburn, Haddow and Gillett, 1948). Special methods employed in the present work are given here.

Titrations of virus content of tissues.

Tissues were removed aseptically and weighed, and 10 per cent suspensions were prepared in 10 per cent non-immune serum in physiological saline (hereafter referred to as serum-saline). All suspensions for use in a given test were centrifuged simultaneously for 20 minutes at approximately 3000 r.p.m. Serial decimal dilutions of the supernate from each suspension were prepared in serum-saline. The same flask of diluent was used for all the tissues in any one experiment. Groups of 6 or 12 mice were inoculated with each dilution of each suspension. Preparation of the dilutions was timed to coincide with the rate of inoculations. All inoculations were done by the same technician. End-points were calculated by the method of Reed and Muench (1938).

Use of modified virus as a vaccine.

In mice.—All the mice to be used in a given experiment were brought into the laboratory at the same time, and separated into test and control groups which were as nearly alike as possible in age and sex. The mice of one group were then inoculated intraperitoneally, each with 0·06 ml. of a 1 per cent brain suspension of mice infected with intracerebral-passage virus (Smithburn, Haddow and Gillett, 1948), strain 2. At selected intervals after the immunizing injections, intraperitoneal titrations of fully virulent pantropic virus were made in both the control and vaccinated mice, using 12 mice per group in each instance.

In lambs.—European-native hybrid stock lambs were used. Prior to the experiments a protection test was done on each to make certain it was non-immune. In each test the immunized and control animals were as near the same age as possible, and in one instance they were twins. Single injections of 1·0 ml. of 1 per cent or 10 per cent mouse-brain supernate were given subcutaneously. Following the immunizing injections, each lamb was bled daily, and its serum was inoculated intracerebrally into mice to test for the presence of the neurotropic virus in the blood. Protection tests were also made at various intervals after the injection of neurotropic virus to learn whether antibody was demonstrable in the serum. At an appropriate interval following the inoculation of neurotropic virus, the test and control lambs were inoculated subcutaneously with a challenge dose of virulent pantropic Rift Valley fever virus. Daily tests for circulating virus were made in both the test and control animals. Their sera were, in this case, inoculated intraperitoneally into mice. Temperatures were taken on lambs once daily on Sundays and holidays, and twice daily at other times. All lambs remaining alive at the end of the experiments were bled, and protection tests were done on their sera.

EXPERIMENTS AND RESULTS.

The first indication that our stock intracerebral-passage virus had become modified was obtained when it was noted that the titre of virus in intraperitoneal protection tests was lower than formerly, that those mice which sickened and died did so only after prolonged incubation periods, and that there was a considerable incidence of paralysis among mice inoculated either intraperitoneally or intracerebrally. Paralysis had not been observed at all in the earliest passages, even when mice were inoculated intracerebrally. In fact, a striking feature was the paucity or total absence of lesions in the brains of mice

which received intracerebral inoculations with low passage virus. The pathogenic effects were manifest wholly or principally in the abdominal visceral tissues, notably in the liver. By contrast, histological examinations of mice receiving high intracerebral-passage virus revealed a complete absence, or striking diminution in the extent, of hepatic lesions, and the appearance of necrosis in nerve cells in the brains of the mice.

Virus lines of low, medium and high intracerebral passages were on hand at this time. These were employed in experiments to determine the concentration of virus in various tissues at different passages, and to learn whether there were marked quantitative differences in the potency of virus introduced by different routes. The tests with low, medium and high passage virus were made on different days, within a 2-week period. In each experiment 2 sick mice were sacrificed, and titrations were made of the virus content of the pooled hearts, lungs, spleens, kidneys, livers and brains.

As shown in Table I, the low passage virus was present in high concentration in all the tissues, possibly owing to its presence in the blood of the mice. The

TABLE I.—*Results of Intracerebral and Intraperitoneal Titrations of Virus Content of Various Tissues of Mice Inoculated with Low, Medium or High Passage Mouse-brain Virus.*

Tissue.	Route of inoculation.	Titre of virus in tissue, 1 in—		
		9th passage.	Medium* passage.	79th passage.
Heart	Intracerebral	31,700,000	616,000	< 10
Lung	,,	72,500,000	427,000	1,380
Spleen	,,	72,500,000	850,000	537
Kidney	,,	49,000,000	575,000	42
Liver	,,	83,000,000	31,700,000	100
,,	Intraperitoneal	16,300,000	15,800,000	< 10
Brain	Intracerebral	252,000	1,520,000	16,300,000
,,	Intraperitoneal	16,300	10,300	370

* 9th intracerebral passage of virus recovered from a human who was suffering from a laboratory infection probably induced by 22nd intracerebral passage of stock virus strain 2, isolated from mosquitoes. This probably represents 31st mouse passage with one intervening human passage.

concentration of virus was highest in the liver and lowest in the brain. The intraperitoneal titration of the brain gave a lower value than did the intracerebral titration, a difference which is probably significant, in view of the fact that each dilution was tested by each route in groups of 12 mice.

The medium passage virus showed significantly lower titres in all tissues except liver and brain. The values for liver were actually less, but probably not significantly so. The intracerebral titration of medium passage brain gave a higher value than did the low passage virus, but the intraperitoneal titration of medium passage brain showed a relative, if not an absolute, decline in potency.

The 79th passage mouse tissues showed consistently and significantly lower virus titres in every instance except in the brain titrated intracerebrally. The latter tissue showed a significant rise in virus content. However, comparison of the intracerebral and intraperitoneal titrations reveals that, although the brains contained a great deal of virus, it was not very effective when introduced intraperitoneally.

These studies made it obvious that the virus had undergone a progressive modification during the course of serial intracerebral passages. It seemed

important to determine whether this was an accidental phenomenon, or whether it occurred regularly, and could be induced under controlled conditions. Accordingly, an ampoule of desiccated 7th intracerebral-passage virus, known to possess high hepatotropic potency, was rehydrated, and groups of mice were inoculated intracerebrally and intraperitoneally. Employing brain tissue, serial intracerebral passages were made from the group inoculated intracerebrally; and from the group inoculated intraperitoneally serial intraperitoneal passages of liver suspension were made. Tissues of mice in each of these series were studied histologically at various passage intervals. Further, intracerebral and intraperitoneal titrations of 9th intracerebral-passage mouse livers and brains were made, and intracerebral and intraperitoneal titrations of livers and brains of both passage lines were made at the 20th and 61st transfers. The titrations of 9th-passage mouse tissues formed a part of the previous experiment.

The results of these tests, shown in Table II, indicate that the decline in hepatotropism with serial intracerebral passage of Rift Valley fever virus was

TABLE II.—*Results of Tests Showing Loss of Hepatotropism and Enhancement of Neurotropism with Serial Intracerebral Passage, and Maintenance of Hepatotropism with Passage of the Same Strain Intraperitoneally.*

Passage line.	Tissue tested.	Route inoculated.	Titre of virus, 1 in— 9th passage.	20th passage.	61st passage.
Intracerebral passages employing brain tissue	Brain	Intracerebral	252,000	56,000	1,580,000
		Intraperitoneal	16,300	14,200	< 10*
	Liver	Intracerebral	83,000,000	31,700,000	32
		Intraperitoneal	16,300,000	1,420,000	< 10*
Intraperitoneal passages employing liver tissue	Brain	Intracerebral	..	6,900	56,000
		Intraperitoneal	..	5,000	100,000
	Liver	Intracerebral	..	31,700,000	317,000,000†
		Intraperitoneal	..	23,000,000	317,000,000†

* 10 per cent suspension caused neither illness nor death.
† End-points not obtained.

not an accidental phenomenon. It occurred again and under controlled conditions. There was, in addition, evidence of simultaneous enhancement of neurotropism with serial intracerebral passage. On the other hand, hepatotropism (or pantropism) was well maintained by intraperitoneal transfer of liver tissue made through an equal number of passages and over the same period of time.

Histological studies.

Tissues from mice of the earliest passages, at which time paralysis was not a part of the clinical picture, exhibited the following features:

Brain.—Neither gross nor microscopic lesions attributable to the virus were present.

Liver.—This tissue was the site of the principal lesions. Usually the liver was brownish red in colour and less firm than normal, but not increased in size. Congestion was intense and haemorrhages were common. The hepatic cells were involved in a pan-lobular necrosis of extreme degree. Many hepatic cells showed swollen, vesicular nuclei with chromatin arranged around the nuclear membranes; some cells so affected contained intranuclear acidophilic inclusions

of round, oval or irregular form. Other cells exhibited pyknosis or karyorrhexis. Nuclear debris was abundant. Less affected cells showed only cloudy swelling, but very few hepatic cells escaped injury. The hepatic cords were disrupted. Small, round or oval, brightly acidophilic "hyaline pearls" were scattered through the tissues, some apparently in sinusoids, others in the cytoplasm of hepatic cells. In the occasional mice which survived longer than the average, the debris of the mass of necrotic cells was in the process of being engulfed by large, highly vacuolated phagocytes, probably Kupffer cells.

A systematic study of the progressive changes occurring during the neuro-adaptation of Rift Valley fever virus has been made by S. F. Kitchen. It suffices here to record the lesions found in high intracerebral-passage mice, and to compare these with the lesions induced by the early-passage pantropic virus.

Liver.—Tissue from a mouse of the 67th passage showed minimal evidence of the lesions described above. No hepatic lesions were observed in 78th- and 81st-passage mice.

Brain.—The principal lesions in mice of the 67th, 78th and 81st intracerebral passages were in the brain. These consisted of congestion, diffuse necrosis of ganglion cells with derangement of their chromatin pattern (in the 67th passage, most pronounced in Ammon's horn) and slight diffuse infiltration.

Thus, concurrently with the loss of virulence by intraperitoneal inoculation, the intracerebral-passage virus had lost all, or nearly all, its capacity to produce lesions in the liver, and had acquired the ability to produce lesions in the brain.

Experiments on Immunization with the Neurotropic Virus.

The pathogenicity of the virus for abdominal visceral tissues had been so reduced by continued brain-to-brain passage that intraperitoneal introduction of the agent seldom resulted in death. Consequently, it was decided to study its capacity to induce an effective immunity. Two tests were made in mice and 4 in lambs.

Tests of immunizing potency in mice.

The first experiment in mice employed 4 groups of vaccinated animals and 2 groups of non-vaccinated controls. One group of mice received 2 intraperitoneal immunizing injections of the neurotropic virus spaced by an interval of 1 week. Three other groups of mice received single intraperitoneal injections of the same virus. The vaccinating dose was 0·06 ml. of 1 per cent brain suspension. The virus used on the first occasion was the unfiltered supernate of an 83rd intracerebral-passage mouse brain suspension. That used on the second occasion was the Seitz filtrate of an 85th intracerebral-passage mouse brain suspension. Twelve mice out of the 401 in the 4 vaccinated groups died in the interval of 3 weeks between the first injections and the challenge inoculations. Two out of 201 unvaccinated controls died in the same period.

Three weeks after the first and 2 weeks after the last immunizing injections all the surviving vaccinated and control mice were inoculated intraperitoneally with pantropic virus. The challenge inoculum was a Seitz filtrate of a suspension of the livers of 2 sick mice of the 62nd intraperitoneal liver-passage group. In order to determine the average survival time, 22 to 28 mice of each vaccinated or control group were given intraperitoneal injections of 0·06 ml. of 1 in 100,000

liver suspensions. The remainder of the mice in each group were used for titration of the challenge virus, with 12 of each group receiving each virus dilution. Results of the test are shown in Table III.

TABLE III.—*Results of Challenge Inoculations with Pantropic Rift Valley Fever Virus in First Experiment on Immunization of Mice with the Neurotropic Virus.*

Group.	Date born.	Date vaccinated.	Sex.	Virus dilution, log.	Fate of mice. Died.*	Fate of mice. Lived.	Titre of virus, 1 in—	Average survival time (days).
A	1–3 July	12 August	F.	4	0	12	< 10,000	10+
			F.	5	0	12		
			F.	6	0	11		
			F.	7	0	12		
			F.	8	1	11		
			3F., 9 M.	9	0	11		
			M.	5	0	22		
B	1–3 „	12 „	F.	4	0	8	< 10,000	10+
			F.	5	0	8		
			F.	6	0	6		
		19 „	F.	7	1	7		
			F.	8	0	12		
			7F., 5M.	9	0	11		
			M.	5	0	28		
C	1–3 „	19 „	F.	4	0	12	< 10,000	10+
			F.	5	0	12		
			F.	6	0	12		
			F.	7	0	12		
			11F., 1M.	8	0	12		
			M.	9	0	12		
			M.	5	0	23		
D	9 „	19 „	F.	4	0	12	< 10,000	10+
			F.	5	0	12		
			F.	6	0	12		
			F.	7	0	12		
			4F., 8M.	8	0	12		
			M.	9	0	12		
			M.	5	0	27		
E	1–3 „	Controls, not vaccinated	F.	4	12	0	56,000,000	1·81
			F.	5	12	0		
			F.	6	12	0		
			F.	7	12	0		
			F.	8	2	10		
			6F., 6M.	9	3	9		
			M.	5	27	0		
F	9 „	Ditto	F.	4	12	0	32,400,000	2·57
			F.	5	12	0		
			F.	6	11	1		
			F.	7	10	2		
			11F., 1M.	8	3	9		
			M.	9	0	12		
			M.	5	26	2		

* Deaths in the first 24 hours are disregarded as being probably due to causes other than specific infection.

In this experiment the protection afforded mice by the immunizing injections of neurotropic virus was nearly absolute. Considering the 2 control series of mice, E and F, as one group, the maximal test dose of virus was 4380 LD_{50}.

The vaccinated mice of groups A, C and D resisted this and smaller doses completely. The test dose of virus in the groups used for determination of the mean survival time was 438 LD_{50}. There were only 2 survivors out of the 55 non-vaccinated mice of series E and F used for this purpose. The average survival time was 2·2 days. By contrast, there were no deaths among the 100 mice of the 4 vaccinated groups receiving the same inoculum.

There were 20 deaths among the 101 mice of series B within the first 24 hours after challenge inoculation (these are not shown in Table III as they were not caused by the specific pathogenic action of the virus), whereas there were only 2 deaths in the same period among the remaining 369 mice which received the challenge inoculation. The mice of series B were the only ones which received more than 1 immunizing injection, and these early deaths following the challenge inoculation may have been due to allergic reactions. An experiment was subsequently done to test this possibility; although the result gave indication that hypersensitiveness was involved, the point was not proved beyond doubt.

Having found that mice are protected against challenge inoculation with pantropic virus within 2 weeks after a single intraperitoneal injection of the relatively innocuous (by this route) neurotropic virus, a second experiment was done to determine how early this protection is manifest. A large group of mice was inoculated intraperitoneally, each with 504,000 intracerebral LD_{50} of a Seitz-filtered 1 per cent mouse brain suspension of the 83rd intracerebral passage. Although the virus used to immunize in this experiment was of the same passage as that used in the first experiment in mice, it was of a different line, which was likewise derived from desiccated 7th passage virus, and likewise observed to have undergone a similar neurotropic modification with continued intracerebral passage in series.

The neurotropic virus used in this experiment was more lethal by intraperitoneal inoculation than that used in the first experiment. Thirty-three of the 348 mice receiving it died as a result; only 1 of 313 unvaccinated controls died during the same period. In addition, there were, among the 6 series comprising 315 vaccinated mice, 14 scattered deaths following challenge inoculation. Some of these may have been caused by the neurotropic virus. The mortality was thus about 3 times as high in this series of vaccinated mice as in the first series.

At intervals of 4, 7 and 10 days after the immunizing injections of neurotropic virus, pantropic virus was titrated in groups of 6 vaccinated and 6 control mice. Fourteen days after the single immunizing injections, pantropic virus was titrated intraperitoneally in groups of 12 vaccinated and 12 normal mice; neurotropic virus was also titrated intracerebrally in both vaccinated and normal mice. At the same time, special groups were also inoculated with each virus to determine the average survival time.

The results of this experiment are shown in Table IV, which indicates that the intraperitoneal inoculation of mice with neurotropic virus protects these animals within 4 days against inoculation with pantropic virus. The most concentrated dilution of virus inoculated in the 4th-day test contained 31,700 LD_{50} of virus, yet there were no deaths among the vaccinated mice receiving this dose of challenge inoculum. Similar results were obtained in the 7th, 10th and 14th day post vaccination challenges with pantropic virus.

The fact of induced resistance is further emphasized by the data on average

TABLE IV.—*Condensed Protocol of Results of the Second Experiment on Immunization of Mice with Neurotropic Rift Valley Fever Virus, followed by Challenge Inoculations with Pantropic and Neurotropic Virus.*

Vaccinated or controls.	No. of mice per group.	Interval between vaccination and challenge (days).	Challenge virus.	Route of inoculation of challenge virus.	Titre challenge virus, 1 in—	Average survival time (days).*
Vaccinated	6	4	Pantropic	Intraperitoneal	< 10,000	..
Controls		..			317,000,000	..
Vaccinated	6	7	"	"	< 10,000	..
Controls		..			142,000,000	..
Vaccinated	6	10	"	"	< 10,000	..
Controls		..			1,000,000,000	..
Vaccinated	12	14	"	"	< 10,000 .	10+ (17,800)†
Controls		..			178,000,000 .	1·06 (17,800)
Vaccinated	12	14	Neurotropic	Intracerebral	14,800 .	9·26 (100)
Controls		..			1,000,000 .	3·51 (100)

* Based on 29 to 33 mice per test.
† Figures in parentheses indicate LD_{50} of virus inoculated for the test of average survival time.

survival time in vaccinated and control mice challenged with either pantropic or neurotropic virus, and by the results of titrations of neurotropic virus in vaccinated and control mice. The latter result is especially significant in view of the fact that the intracerebral challenge with neurotropic virus is a very severe test.

Tests of immunizing potency in lambs.

Two lambs were used in the first experiment. When these animals were obtained they were said to be about 5 months old. They had been in our hands 3 months when these experiments were done. In view of the result in Lamb 5, referred to later, it seems highly probable that this one, at least, was somewhat older than stated. Lamb 4 was inoculated subcutaneously with 1·0 ml. of unfiltered 1 per cent mouse brain suspension of the 83rd intracerebral passage of Rift Valley fever virus. On the 21st day following the inoculation with neurotropic virus, this animal and the unvaccinated control, Lamb 5, were each inoculated subcutaneously with 1·0 ml. of 1 in 100,000 dilution of pantropic Rift Valley fever virus (61st intraperitoneal liver passage in mice). The dose for each lamb proved to be 7314 intraperitoneal LD_{50}.

All the mice inoculated intracerebrally with serum taken from Lamb 4 during a period of 10 days after inoculation with the neurotropic virus survived. Therefore there was no demonstrable circulating virus. Lamb 4 had irregular temperatures prior to this inoculation, once as high as 105·6° F., and on several occasions up to 104·6° F. On the morning following the injection of the neurotropic virus its temperature was 104·6° F., and on the 7th day its afternoon temperature was 105·0° F. On 6 other occasions during the 10-day period after inoculation with the neurotropic virus its temperature was between 104·0 and 104·6° F. It is not clear whether this represented a febrile reaction or not,

but if so the reaction was not marked. The animal continued to eat normally and at no time appeared ill.

Table V shows the mortality ratios in mice used to test the sera of lambs 4 and 5 for circulating pantropic virus on the 2nd to 10th days following the challenge inoculation.

TABLE V.

Days after challenge.	Lamb 4 (vaccinated).	Lamb 5 (normal control).
2	0/6	6/6
3	0/6	6/6
4	0/6	6/6
5	0/6	0/6
6	0/6	0/6
7	0/6	0/6
8	0/6	0/6
9	0/6	0/6
10	0/6	0/6

Numerator indicates number of mice which died; the denominator indicates the number inoculated.

No tests for circulating virus were made on the first day after inoculation with pantropic virus. Lamb 5, the non-vaccinated control, showed circulating virus from the 2nd to 4th day, but none thereafter. All tests on the vaccinated lamb were negative.

Lamb 4, the vaccinated animal, had temperatures of 105·0° and 105·6° F. on the afternoons of the 1st and 4th days respectively after inoculation with pantropic virus, but its temperature was within normal limits at other times during the 10-day observation period. It is possible that this represents a mild saddleback elevation. However, the temperatures in this animal were so variable that it is not certain that it had a febrile response. Lamb 5, on the other hand, had a fairly stable preinoculation temperature record, with a maximum normal of 104·2° F. Forty-eight hours after inoculation with pantropic Rift Valley fever virus its temperature rose sharply to 106·2° F. Its temperature was normal the next morning, and remained so. It is noteworthy that, although the animal was afebrile on the 3rd and 4th days after inoculation, it had circulating pantropic virus on both those days.

Results of the protection tests on Lambs 4 and 5, shown in Table VI, demonstrate that Lamb 4 developed protective humoral antibody following inoculation with the neurotropic virus, and that it had a further rise in antibody following inoculation with the pantropic virus. Whether the antibody response to the neurotropic virus was submaximal, or whether the interval between the 2 inoculations did not permit a complete response to the neurotropic before inoculation of the pantropic virus, cannot be determined from this result. In other experiments, however, we have found evidence to indicate that the neurotropic virus is a less efficient antigen than the pantropic. Nevertheless, that it does induce antibody formation is shown by the results in Table VI.

An interesting incidental observation was made during this experiment. Unknown to us, Lamb 5 was pregnant at the time of the experiment. On the

TABLE VI.—*Results of Intraperitoneal Rift Valley Fever Protection Tests on Sera of a Vaccinated Lamb at Various Intervals after Inoculation with Neurotropic Virus, and on Sera of This and the Control Lamb after Challenge Inoculation with Pantropic Virus.*

Serum.	Days after vaccination.	Days after challenge inoculation.	Test virus dilution, log.	Fate of mice. Died.	Fate of mice. Lived.	Titre of virus, 1 in—
Lamb 4, preinoculation	0	..	5	6	0	31,700,000
			6	5	1	
			7	4	2	
			8	2	4	
			9	1	5	
August 22	10	..	5	6	0	2,520,000
			6	5	1	
			7	0*	6	
September 2	21	..	5	6	0	1,000,000
			6	3	3	
			7	0	6	
„ 11	30	9	5	2	4	< 100,000
			6	0	6	
„ 19	38	17	5	2	4	< 100,000
			6	0	6	
Lamb 5, preinoculation	..		5	6	0	23,000,000
			6	6	0	
			7	4	2	
			8	1	5	
			9	0	6	
September 10	..	8	5	6	0	317,000
			6	0	6	
„ 19	..	17	5	0	6	< 100,000
			6	0	6	
Lamb 3, immune control	..	..	2	1	5	< 100
			3	0	6	

* Additional tests with higher dilutions beyond the end-points with this and other sera are omitted.

7th day after inoculation she aborted a nearly full-term foetus. This animal was said to be about 5 months old when it was received. It had been in our possession 3 months when this experiment was done. Little is known about the age of maturity of native or European-native hybrid sheep, but in view of its pregnancy it seems probable that Lamb 5 was older than we were led to believe. Serum and a suspension of liver from the foetus were inoculated into mice. Virus was recovered from both, and in each instance was identified by a protection test as Rift Valley fever virus. The most interesting point in this connection is that the virus which traversed the placenta was present in both the blood and the liver of the foetus 72 hours following the last positive test on the serum of the mother.

In the second experiment one weaned (No. 14) and 2 unweaned (No. 13 and 15) lambs were used as controls. Another weaned (No. 16) and 2 unweaned (No. 11 and 12) lambs were inoculated subcutaneously with 83,916,000 mouse-intracerebral LD_{50} of 83rd intracerebral-passage virus. All the tests for circulating virus were negative, showing that there was no demonstrable neurotropic virus in the blood of either of the lambs during the 14 days following inoculation. Lamb 11 had no temperature reaction whatever to the inoculation of neurotropic virus. Lamb 12 had a temperature of 104·3° F. 30 hours after vaccination and normal temperatures at other times. Normal maximum for this lamb was 103·4° F. Weaned Lamb No. 16 had temperatures of 104·3° F.

in the first 3 days after the inoculation, and normal temperatures thereafter. These reactions probably represent mild febrile responses in Lambs 12 and 16.

Three weeks after the immunizing injections these animals, together with their non-vaccinated controls, were inoculated subcutaneously, each with 2,922,500 mouse-intraperitoneal LD_{50} of pantropic virus. The non-vaccinated lambs showed virus in their sera for 2, 3 and 4 days, but not thereafter, while the vaccinated lambs gave consistently negative results, as may be seen from Table VII.

TABLE VII.—*Mortality Ratios in Mice Inoculated with Sera of Lambs.*

Day after inoculation.	Vaccinated lambs, No.			Normal lambs, No.		
	11	12	16	13	14	15
1	0/6	0/6	0/6	6/6	6/6	6/6
2	0/6	0/6	0/6	6/6	5/6	6/6
3	0/6	0/6	0/6	6/6	0/6	5/6
4	0/6	0/6	0/6	6/6	0/6	0/6
5	0/6	0/6	0/6	0/6	0/6	0/6
6 to 10	All tests negative for virus.					

Two of the vaccinated lambs (No. 11 and 12) had no febrile reactions following inoculation with the pantropic virus. Lamb 16 had a temperature of 104·4° F. on the 7th day following inoculation, but had normal temperatures at other times. Each of the 3 non-vaccinated lambs had a sharp temperature reaction of 1 to 4 days' duration. The peaks, 106·2° to 107·6° F., in each instance occurred on the second post-inoculation day.

All the control lambs in this experiment survived. The demonstration of the immunizing action of the neurotropic virus therefore depended on the temperature reactions, the tests for circulating virus, and the protection tests with serum specimens taken prior to the challenge inoculation with pantropic virus.

In Table VIII, showing results of the protection tests, it is obvious that Lambs 11, 12 and 16 had specific antibody responses to the inoculation of neurotropic virus. Lambs 11 and 16 exhibited further rises in antibody titre following challenge inoculation with the pantropic virus. Each of the control lambs likewise had protective antibody in its serum 14 days after injection of the pantropic virus.

Up to this point the experiment was wholly successful, and gave complete confirmation of the first experiment. It shows that the neurotropic virus is efficacious as a vaccine, and that it is not followed by severe reactions, nor by the appearance of a demonstrable quantity of the neurotropic virus in the circulation. However, the fact that the 3 vaccinated lambs died from undiscovered cause on the 8th, 25th and 33rd days after challenge inoculation with pantropic virus marred the experiment. Prior to death each animal became very weak, had subnormal temperature and passed large bulky stools. There was no paralysis, and the animals were able and willing to eat when food was placed before them. Subinoculation of blood and suspensions of their brains to mice yielded nothing. Gross autopsies and microscopic examinations failed to show a cause of death. The only lesions of consequence were moderate degenerative changes in the renal tubules, the presence of albuminous deposits in their lumina, and intestinal

TABLE VIII.—*Results of Intraperitoneal Rift Valley Fever Protection Tests Showing Development of Protective Antibody in Lambs of the Second Experiment.*

Lamb No.	Serum taken. Date.	Days after neurotropic.	Days after pantropic.	Titre of virus, 1 in—
5 normal control	2 Sept., 1946	..	..	100,000,000
11	13 Nov., preinoculation	..	..	25,200,000*
11	6 Dec.	21	..	560,000
11	20 „	35	14	< 100,000†
12	13 Nov., preinoculation	..	..	31,700,000*
12	6 Dec.	21	..	< 100,000†
12	13 „	28	7	252,000
16	13 Nov., preinoculation	..	..	31,700,000*
16	6 Dec.	21	..	2,520,000
16	20 „	35	14	< 100,000†
13	6 „ preinoculation	..	..	31,700,000*
13	20 „	..	14	< 100,000†
14	6 „ preinoculation	..	..	6,900,000*
14	20 „	..	14	100,000
15	6 „ preinoculation	..	..	31,700,000*
15	20 „	..	14	< 100,000†
3 immune control	1 July, 1946	..	..	100

* 1 in 10,000,000 virus was the weakest dilution inoculated and complete end-points were not obtained.

† 1 in 100,000 virus was the strongest dilution inoculated and serum was protective against this.

worms in 2 of the animals. During the experiment all the lambs were kept in an insect-proof room with cement floor and were fed solely on grass. It is possible that the 3 lambs succumbed from inanition, as the vaccinated animals were bled daily for 2 weeks longer than the controls. The cause of death was not discovered. There was no evidence that either the neurotropic or the pantropic virus was responsible for the fatal issue. In fact, each of the animals had protective antibody in its serum prior to inoculation with the pantropic virus, and exhibited little or no reaction to that agent.

Two sets of twin lambs were available for two further experiments on immunization. However, the first test could not be completed, because the control lamb died from undiscovered cause prior to challenge inoculation.

The twin Lambs 19 and 20 were born March 30, 1947, to a non-immune ewe. Five days later both were bled for protection tests, and Lamb 19 was inoculated subcutaneously with 67,200,000 mouse-intracerebral LD_{50} of 98th-passage neurotropic Rift Valley fever virus. The lamb was bled daily for 10 days from the jugular vein, and its whole blood was inoculated intracerebrally into mice to test for circulating virus. All the inoculated mice remained well, so no evidence was obtained that the virus entered the blood. The lamb had no temperature reaction to the inoculation and exhibited no objective signs. A protection test made on serum taken from this animal 21 days after inoculation of the neurotropic virus showed that it had developed protective antibody as a result of the procedure. Its twin, intended for use as control for the challenge inoculation, having died, the test inoculation of Lamb 19 was not done. Nevertheless, the experiment showed that new-born lambs may be safely inoculated with the neurotropic virus, and that they develop neutralizing antibody as a result.

Lambs 21 and 22 were also twins born to a non-immune ewe. When they were 4 days old both were bled for protection tests, and No. 21, a male, was

inoculated subcutaneously with 1,781,000 mouse-intracerebral LD_{50} of 100th-passage neurotropic virus. Lamb 22, a female, was reserved as the control for the challenge inoculation.

The tests for circulating virus in Lamb 21 in the 10 days following inoculation with neurotropic virus were negative for that virus. However, another agent pathogenic for mice but not neutralized by potent Rift Valley fever immune serum was present in the blood of the lamb for the first 4 days. This agent caused death in mice in 5 to 7 days ; it was not identified. Lamb 21 had no elevation of temperature during the 3 weeks following the inoculation of neurotropic Rift Valley fever virus. Neither the latter nor the unidentified agent in its circulation which was pathogenic for mice sufficed to induce a febrile reaction. Serum taken from Lamb 21 on the 18th day after the immunizing injection contained abundant neutralizing antibody against pantropic Rift Valley fever virus.

On the 39th day after the inoculation of Lamb 21 with the neurotropic virus, Lambs 21 and 22 each received subcutaneously a challenge inoculation of 158,500 mouse-intraperitoneal LD_{50} of pantropic Rift Valley fever virus. The vaccinated lamb had no temperature exceeding 104·0° F. following the challenge inoculation, and daily tests for circulating virus were negative. The non-vaccinated twin control, Lamb 22, had a sharp rise in temperature to 105·0° F. 48 hours after inoculation, but its temperature was normal the following day, and remained so. The tests in mice for circulating virus in Lamb 22 showed that Rift Valley fever virus was present in the blood during the first 72 hours after the test inoculation. Thirteen days after the inoculation with pantropic virus the sera of Lambs 21 and 22 both gave complete neutralization in tests against 560,000 LD_{50} of pantropic virus.

Inherited Immunity Against Rift Valley Fever.

Daubney and Hudson (1931) pointed out that very young lambs are much more susceptible to Rift Valley fever than older ones. In order to protect lambs by active immunization through their period of maximal susceptibility, it would be necessary to inoculate them very soon after birth. This might not only be impractical, but might also be associated with some hazard. Another approach to the problem would be to immunize the ewes, provided they passed on the immunity to their offspring, and provided the inherited immunity were sufficiently enduring to be of practical importance.

During the course of our investigations an opportunity was afforded to study the duration of the humoral immunity inherited by lambs born to an immune ewe. On December 31, 1946, Rift Valley fever immune Ewe No. 1 delivered twin male lambs which were given numbers 17 and 18. Fifteen days after they were born the sera of these lambs were both found to be protective against pantropic Rift Valley fever virus. When they were 1 month old Lamb 17 was taken from the ewe and fed thereafter on cows' milk and grass. Lamb 18 remained with the mother ; it fed on grass and continued to suckle. Each animal was bled once a week from the time they were 38 days old until they were 171 days old, and protection tests were done on their sera.

The data on these tests were very voluminous, and only a selected few are presented in Table IX. In the earliest tests made, the sera of Lambs 17 and 18 contained sufficient antibody to protect against 1000 or more mouse-intra-

peritoneal LD_{50} of pantropic Rift Valley fever virus. By the time they were 11 weeks old the antibody titre in both animals had declined to the point where quantitative tests employing several dilutions of virus had to be made in order to determine whether antibody was present. However, antibody sufficient to neutralize 10 LD_{50} of virus was still present in the serum of the unweaned lamb

TABLE IX.—*Selected Results from Serial Weekly Protection Tests on Lambs Born to an Immune Ewe.*

Serum of Lamb No.	Age (days).	Weaned (weeks).	Virus dilution, log.	Fate of mice. Died.	Fate of mice. Lived.	Average survival time (days).	Titre of virus, 1 in—
Normal control	45	2	5	6	0	1·3	
17	45	2	5	1	5	9·2	
18	45	2	5	0	6	10·0	
Immune control	45	2	5	0	6	10·0	
Normal control	..	..	4	6	0	1·3	30,200,000
			5	6	0	1·2	
			6	6	0	2·0	
			7	4	1	4·0	
			8	1	5	8·7	
			9	0	6	10·0	
17	129	14	4	6	0	2·0	3,170,000
			5	6	0	2·0	
			6	5	1	5·0	
			7	1	5	8·8	
			8	0	6	10·0	
18	129	..	4	5	1	3·2	317,000
			5	5	1	3·5	
			6	1	5	8·7	
			7	1	5	8·7	
			8	0	6	10·0	
Immune control	..	..	1	0	6	10·0	< 10
			2	0	6	10·0	
			3	0	6	10·0	
			4	0	6	10·0	
Normal control	..	..	5	6	0	1·7	56,000,000
			6	6	0	1·7	
			7	6	0	2·0	
			8	2	4	7·3	
			9	0	6	10·0	
17	150	17	5	6	0	2·0	3,170,000
			6	4	2	5·8	
			7	2	4	7·5	
			8	0	6	10·0	
18	150	..	5	5	1	3·2	10,000,000
			6	5	1	3·2	
			7	4	2	5·7	
			8	0	6	10·0	
17	157	18	5	6	0	2·0	10,000,000
			6	6	0	2·8	
			7	3	3	7·2	
			8	0	6	10·0	
18	157	..	5	6	0	1·7	20,500,000
			6	6	0	2·0	
			7	3	3	6·2	
			8	2	4	7·7	
Immune control	..	..	1	0	6	10·0	< 10
			2	0	6	10·0	
			3	0	6	10·0	
			4	0	6	10·0	

Horizontal rulings demarcate different tests with separate controls.

143 days after its birth, and in the weaned lamb this amount of antibody was demonstrable on the 150th day.

In the earliest studies made in this experiment there was evidence that the inherited immunity in Lambs 17 and 18 was less effective than in ovines convalescent from experimental infection, in that the neutralizing power of the sera of the former was less. Nevertheless, the presence of antibody was found to be demonstrable in 2 ways: by the capacity of the serum to prevent the lethal effects of the virus, and by the effect of the serum in prolonging the survival time in mice. In fact, the average survival time proved to be an efficient indicator of the presence of antibody when the quantity of protective substance was inadequate to prevent death in a significant number of mice. For example, in the 6th week after weaning, the serum of Lamb 17 protected only 1 of 6 mice from the lethal action of the virus, yet the average survival time of the group was 4 times that of controls receiving the same amount of virus mixed with non-immune serum. Conversely, a progressively diminishing average survival time was an effective indicator of the decline of immunity. The average survival time of mice receiving 1 in 100,000 suspension of infective liver tissue mixed with serum taken from Lamb 18 in the 7th, 10th, 13th, 16th, 19th and 22nd weeks of life was, respectively, 10·0, 4·3, 3·8, 3·3, 1·8 and 1·7 days.

From the foregoing experiment it was evident that the inherited humoral immunity was lost very gradually, and that weaning had no effect on the rate of disappearance of the antibody from the serum.

DISCUSSION.

The spontaneous adaptation of Rift Valley fever virus to the nervous system upon serial intracerebral passage in mice, with concomitant diminution in its hepatotropic affinities, seems to indicate the presence of thinly-veiled neurotropic affinities in the native, unmodified virus. These neurotropic affinities gain ascendency under circumstances which are favourable for their prompt action and which, at the same time, are not notably unfavourable for the hepatotropic affinities. Whether the reversion of the neurotropic Rift Valley fever virus to the hepatotropic form can be brought about is not evident from these experiments, but as the adaptation to neural tissues is gradual and progressive, one might assume that reversion could take place up to a point, after which it would be progressively less likely. Studies made by S. F. Kitchen indicate this to be the case.

The neurotropic virus in our hands has never become completely innocuous for mice when introduced extraneurally, nor has it been determined whether the relatively small percentage of mice which succumb to extraneural inoculation do so from the effects of the virus on the liver or on the nervous system. Nevertheless, the neurotropic adaptation of the agent results in a profound modification of its affinities for the tissues of lambs, in that the pantropic virus invariably enters the blood, whereas the neurotropic, in our experience, does not. The evidence available furthermore indicates that adaptation of the virus to nervous tissues is associated with a decline in antigenic potency, as the pantropic virus seems to induce a higher titre of antibodies. Nevertheless, sufficient antigenic potency is retained by the modified virus to stimulate the formation of antibody, which not only neutralizes the pantropic virus *in vitro*, but also vitiates its effects *in vivo*.

Adequately comprehensive investigation of the value of neurotropic Rift Valley fever virus as a practical immunizing agent is beyond the scope of this laboratory at present. However, the results obtained to date give a hopeful outlook, and point to the desirability of further laboratory and field investigations. The fact that the age of maximum susceptibility to Rift Valley fever in sheep falls in the earliest days of life (Daubney and Hudson, 1931) poses an administrative problem in the application of any preventive agent, and perhaps raises additional hazard on account of the tender age of the subjects. The observation that immunity to the disease in the ewe is transmitted to her offspring and persists for some weeks in the latter offers a hopeful lead. It is suggested that the inoculation of ewes with the neurotropic virus at, or before, the time of the mating season would induce immunity, which would be transmitted to the offspring, and might suffice to tide the latter over the age of maximum susceptibility. Furthermore, the ewes, being immune, would not be subject to the hazard of abortion which is associated with natural infection with Rift Valley fever.

SUMMARY.

Several strains of Rift Valley fever virus which were passed in mice by serial intracerebral inoculation acquired enhanced neurotropism with concomitant reduction of hepatotropic affinities.

Neurotropic adaptation of the virus could be brought about under controlled conditions by serial brain passage in mice. Parallel serial liver passage resulted in retention of the hepatotropic properties and no enhancement of neurotropism.

The intraperitoneal inoculation of neurotropic Rift Valley fever virus into mice resulted in enhanced resistance to intracerebral inoculation of neurotropic virus and in complete immunity to pantropic virus.

The subcutaneous inoculation of neurotropic Rift Valley fever virus into newborn lambs or older sheep resulted in the formation of specific antibody demonstrable by protection tests on their sera, and of immunity to the effects of inoculation with the pantropic virus. The neurotropic virus was not demonstrable in the circulating blood of lambs or sheep and caused no objective reactions of consequence.

Humoral immunity inherited by twin lambs from their immune mother was demonstrable for about 5 months. Weaning of one of the twin lambs did not appreciably affect the rate of disappearance of the inherited immunity.

Immunization against Rift Valley fever is discussed, and a suggestion is made for the practical application of a vaccine.

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HYPERPLASIA OF THE PARATHYROIDS ASSOCIATED WITH OSTEITIS FIBROSA IN RATS TREATED WITH THIOURACIL AND RELATED COMPOUNDS.

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SINCE the discovery of the goitrogenic action of thiourea, thiouracil and their derivatives (Richter and Clisby, 1942; Kennedy, 1942; MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissel and Tyslowitz, 1943), a vast literature has arisen describing the effects of such compounds on the thyroid, pituitary and other organs of the rat. As far as we are aware, no report has been published describing changes in the parathyroids after the administration of these drugs. It seems reasonable that such changes have been seen by other workers. We ourselves have been familiar with their occurrence since our earlier studies on the goitrogenic action of brassica seeds (Griesbach, 1941), but only recently have long-term experiments provided us with material permitting an appreciation of the changes induced by goitrogenic agents in the parathyroids. The purpose of this preliminary communication is to describe these changes, and the simultaneous occurrence of osteitis fibrosa in rats treated with thiourea, thiouracil or methylthiouracil for long periods.

MATERIAL AND METHODS.

The rats used belonged to two colonies of albino rats, substrains of Wistar rats, imported into this country 10 years ago. No foreign stock had been added to these colonies. Animals of both sexes, 6–8 weeks of age, were chosen. They received, in their drinking water, either thiourea 0·25 per cent, or thiouracil 0·05 per cent, or methylthiouracil 0·05–0·025 per cent. The diet given consisted of bran 30 per cent, pollard 25 per cent, bone meal 15 per cent, pea meal 15 per cent, maize meal 15 per cent. Use was made also of material obtained in Sheffield from rats which had received 8 mg. of allylthiourea daily. These rats belonged either to another substrain of Wistar rats, or were piebald rats fed on a diet consisting of 3 parts skimmed milk powder and 7 parts dried bread, to which cod liver oil and cabbage were added once weekly. Thyroid digest was prepared by T. H. Kennedy by peptic digestion of G.P. thyroid tablets. The daily dose given by stomach-tube or by injection corresponded to 5 μg. d-l-thyroxine. After killing the animals the parathyroids were left in their connection with the thyroid gland and fixed in 10 per cent formol saline or acid Zenker's solution. After fixation in formol saline the bones were decalcified with 5 per cent trichloracetic acid in Susa's fixative and embedded in paraffin. The sections were stained with haematoxylin and eosin, van Gieson or Weigert's

stain. The pituitaries were fixed in sublimate-formalin, sectioned at 2·5μ, and stained according to Martins' modification of the Mallory method (1933).

RESULTS.

Parathyroids.

The parathyroids of animals treated for a long period with antithyroid drugs are frequently so large that their increased size is already apparent at naked-eye inspection. Their colour is a shiny white, with an occasional slightly yellow tinge. On some occasions the parathyroid is quite prominent, sticking out above the surface of the thyroid. The histological study reveals further changes. Normally, at low magnification, the parathyroid appears in haematoxylin and eosin preparations as a dark blue circular or oval area which is sharply defined (Fig. 1). In our control rats the outer border of the parathyroid reaches the capsule of the thyroid and does not bulge out into the surrounding tissue. Only a thin capsule separates the parathyroid from the thyroid. There is little connective tissue inside the gland, and large blood vessels are rarely found besides the main artery. A small cell with a nucleus of elongated shape, rich in chromatin, predominates. The cytoplasm is scanty and basophilic (Fig. 2). The cell borders are not clearly defined in haematoxylin and eosin preparations. Dispersed among these elements are a few cells which have a larger pale cytoplasm and a round nucleus. In our controls dark oxyphilic and so-called water clear cells were not found. The hyperplastic glands show a different picture. Such glands bulge frequently into the loose areolar connective tissue surrounding thyroid and parathyroid (Fig. 3). Sometimes lobe-like projections are seen to extend into the neighbourhood, and the thyroid tissue surrounding the parathyroid may show compression. This is best seen in the animals with inactive thyroids. Here obviously the parathyroids have grown at a much higher rate than the thyroids, the growth of which had been arrested by continued administration of thyroxine in form of a thyroid digest. Fig. 5 illustrates this phenomenon, showing an inactive thyroid which surrounds like a crescent the large parathyroid, the size of which is about equal to that of the thyroid. This picture also indicates the mode of enlargement of the parathyroid by apposition of lobules and by expansion. The capsule of the gland is thickened, and septa divide the parathyroid into several lobes. Blood vessels follow these septa,

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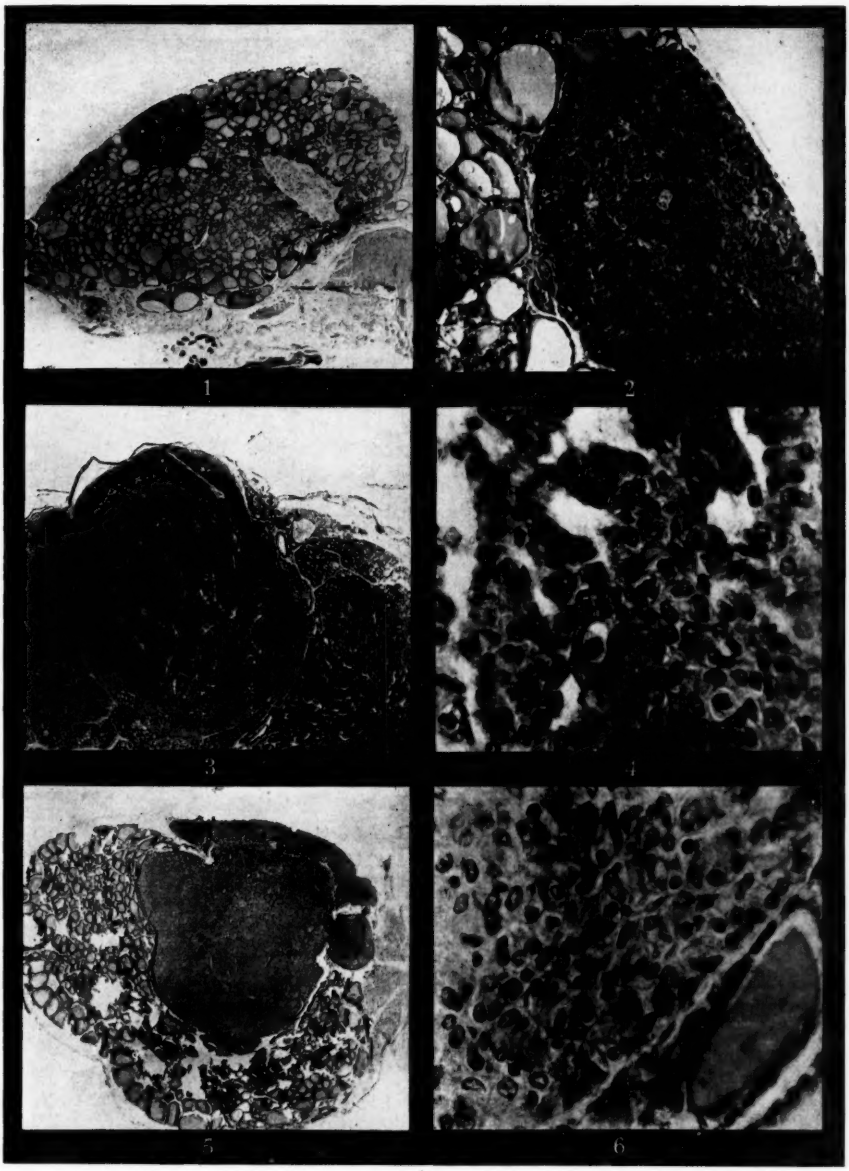
EXPLANATION OF PLATES.

FIG. 1.—Normal thyroid and parathyroid. Note ratio of parathyroid to thyroid size. (× 23.)
FIG. 2.—Normal parathyroid from Fig. 1. (× 100.)
FIG. 3.—Hyperplastic parathyroid and hyperplastic thyroid containing adenomata. (× 23.)
FIG. 4.—Detail from Fig. 3 showing pale oxyphil cells, alveolar arrangement. 2 mitoses. (× 450.)
FIG. 5.—Hyperplastic parathyroid in inactivated thyroid. Note ratio of parathyroid to thyroid size. (× 23.)
FIG. 6.—Cluster of dark oxyphil cells in hyperplastic parathyroid. (× 450.)
FIG. 7.—Transverse section of diaphysis of tibia, showing replacement of compact leg spongious bone. Same rat as in Fig. 3 and 4. (× 15.)
FIG. 8.—Detail of a longitudinal section of tibia. Cellular fibrous marrow, replacing compact bone. Note osteoclast near trabecula. ×(100.)
FIG. 9.—Fibrous marrow containing spindle cells. (× 100.)
FIG. 10.—Osteitis fibrosa. Note numerous osteoclasts. (× 100.)
FIG. 11.—Osteoblasts lining trabecula. (× 450.)
FIG. 12.—Osteitis fibrosa with large haemorrhagic areas. (× 100).



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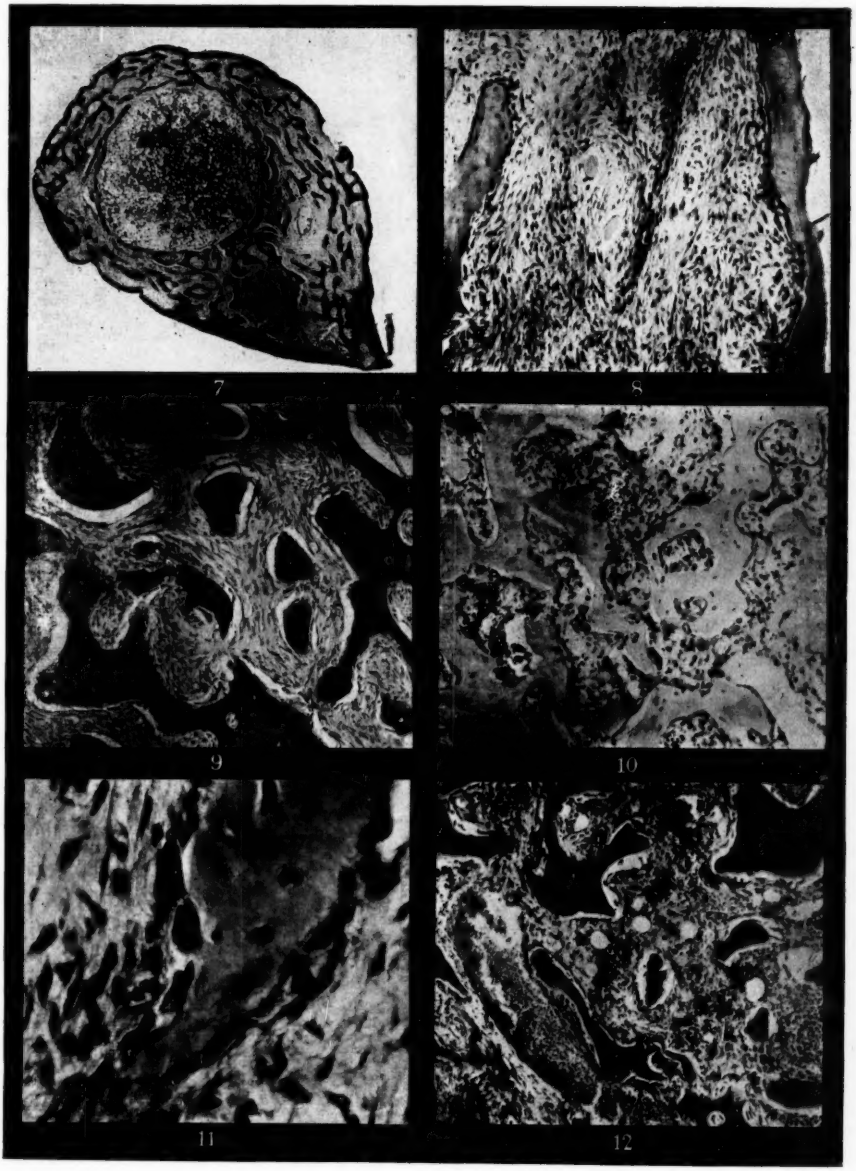

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and frequently a general hyperaemia exists. In normal rats of our strain the longest diameter of the parathyroid in serial sections reaches 0·5–0·7 mm. In the hyperplastic glands diameters up to 2·2 mm. have been found. More frequently were measurements of 1·2–1·6 mm.

At low magnification the staining qualities of the enlarged parathyroids are generally lighter, and resemble more the colour of the surrounding hyperplastic thyroid. At first glance it is obvious that the cytoplasm of the predominating cell type is oxyphilic and the nuclei appear paler. At higher magnification one sees that the amount of cytoplasm is increased, staining a pinkish red, and that the cell borders are sharply defined. These cells are the well-known pale oxyphilic cells (Fig. 4). Their nuclei are round or oval with finely dispersed chromatin, which is displaced towards the quite conspicuous cell membrane. Frequently these cells form alveoli, which are surrounded by delicate strands of connective tissue. Such alveoli may show in the centre rests of pink or purplish colloid-like substance. Exceptionally large accumulations of this colloid were found. Clusters of dark oxyphilic cells with coarsely granulated cytoplasm appear in some of the largest glands (Fig. 6). Mitoses are frequent, especially in the periphery. Here three or more dividing cells can be seen in one field. It seems worth mentioning that in short-term experiments only one of the features described above was present regularly ; besides a moderate general increase of the glands in size, there appeared to be an activation of the parathyroid cells. They were enlarged, and the nuclei were round and lighter in colour, due to the dispersal of chromatin described above. An occasional mitosis was found. In contrast to the experiments carried on for twelve months and more, this early hyperplasia did not occur when thyroid solution was given simultaneously with the goitrogen for 4 or 8 weeks and, consequently, the animals' thyroids were inactive. It seems logical to assume that the early parathyroid hyperplasia is due to the unspecific action of regional hyperaemia induced by the effect of the goitrogen.

Bones.

Once the fact of parathyroid hyperplasia induced by goitrogens was established, a systematic search for changes in the bones was made. Among the many animals studied, only a few were found showing a generalized disease of the skeleton (Table I). All of these belong to the groups of rats which had received the goitrogen for considerable periods; bone changes have never been found in short-term experiments, i.e. 8–16 weeks. Fig. 7–12 illustrate the essential features of the pathological process. A transverse section of the tibia (Fig. 7) shows the normal fatty marrow with foci of haematopoiesis. The compact bone of the corticalis is replaced by spongy bone. Trabeculae of various size and appearance lie in a fibrous marrow which is sharply defined towards the fatty marrow. The connective tissue forms fibrous strands, which are fairly cellular in some areas (Fig. 8), but show only a few spindle cells in others (Fig. 9). Weigert–van Gieson staining reveals the presence of delicate collagenous fibres. Adjacent to the trabeculae the number of cells present in the fibrous marrow increases, and two types of large cellular elements can be found. Many typical multinuclear osteoclasts are seen, frequently situated in Howard's lacunae (Fig. 10). Further, large osteoblasts surround other trabeculae (Fig. 11), especially those which have a small rim of osteoid tissue. Engorged blood vessels

TABLE I.

Treatment.	No. of rats.	Rats with osteitis fibrosa.	Sex of rats with osteitis fibrosa.	Duration of experiment (weeks).
Thiourea	14	..	..	..
0·25%	..	1	M	71
		1	M	107
Methyl-thiouracil	16	..	..	..
0·01%	..	1	M	65
0·025%	..	1	F	71
0·05%	..	1	M	108
		1	M	122
Methyl-thiouracil	3	..	..	..
0·01% + 5 micrograms		2	M	56
d-l-thyroxine daily (for 52 weeks)		1	M	58

are frequently seen, blood extravasation or signs of old haemorrhages only rarely (Fig. 12). In the trabeculae fibrous bone instead of lamellar bone is prevalent. Haversian systems are rare. The severity of the lesions varies greatly in different parts of the same bone. Sometimes one side of the diaphysis shows a normal corticalis, while only a rim of bone is left on the other side. In the most advanced cases the tibia had become so soft that it could be cut without previous decalcification. In the epiphysis essentially the same changes were found. Nearly all the fatty marrow had been replaced by fibrous tissue, and similar cell changes were present as in the diaphysis. As could be expected in these animals in which growth had stopped, the epiphyseal cartilage formed only a narrow zone. But when the epiphyses of normal rats of corresponding age were studied, it became apparent that the chondromedullary line (Gilmour, 1947) in rats with osteitis fibrosa showed obvious pathological changes. Whereas in the controls small columns of cartilage cells of fairly even size were present in the experimental animals, the fibrous marrow invaded the chondromedullary line. The cartilage cells nearest to the fibrous tissue were swollen, and seemed to undergo degenerative changes. At some points the activated fibrous tissue appeared to break through the zone of cartilage.

The whole process is one of greatly increased resorption of bone which, as the study of less advanced lesions proves, starts in the Haversian canals. New bone is formed at the same time, but active apposition is exceeded by destruction. There is no sign of inhibition of calcification. Osteoid may be seen occasionally forming narrow seams round the calcified core of the trabeculae. However, there is no indication of disturbed intracartilaginous ossification as in rickets.

Pituitary.

The pituitary gland, after long-term treatment with thiouracil or methyl-thiouracil (0·025 and 0·05 per cent), presents uniformly the picture of degranulation of the chromophilic cell types. Therefore, only chromophobe elements

are present containing remnants of the basophil colloidal substance situated in intercellular spaces. Chromophobe cells, characterized as degranulated basophils by the typical situation of the negative image of the small Golgi body, are present in moderate numbers. But "thyroidectomy cells," abundant in the early stages of goitrogen action, are absent. The chromophobe cells generally do not contain enlarged Golgi bodies as after prolonged oestrogen treatment, and there is no evidence of actively secreting cells. Neither thiourea (0·25 per cent) nor methylthiouracil in weaker concentration (0·01 per cent) leads to complete degranulation of the acidophil cells. The pituitaries of the two animals which had received a maintenance dose of thyroxine together with the goitrogen for 12 months showed a cell distribution in the anterior lobe which corresponded to the normal picture. The only deviation worth mentioning was the more alveolar arrangement of the glandular cells round the blood capillaries, into which some basophils could be seen to secrete their contents. The pars intermedia in most of the old experimental animals was definitely enlarged. There was a higher number of cell rows made up by enlarged cells having round, well-staining nuclei. Numerous basophilic elements were distributed amongst the ordinary intermedia cells. An unusual number of cysts were visible, surrounded by the intermedia cells. These cysts appeared to be free of any staining material.

DISCUSSION.

Thyroid hyperplasia as produced by thiourea and related compounds is always accompanied by enlargement of the parathyroids. This could be observed as early as 3 weeks after the beginning of drug treatment. It seemed obvious that this effect was due to an increased blood supply to both the thyroid and the parathyroid. In fact, by simultaneous administration of the goitrogen and thyroxine, it could be shown that the hyperplasia of both organs could be suppressed in short-term (8 weeks) experiments. However, when goitrogen and thyroxine were given together for 12 months or more, a quite different picture resulted. Whereas the thyroids had retained their normal size and activity, the parathyroids increased in size proportionally to the time of treatment, reaching in some cases the size of the thyroid. Therefore the regional hyperaemia cannot be an essential factor in the genesis of parathyroid hyperplasia observed in the long-term experiments.

The study of numerous animals has convinced us that hyperplasia of the parathyroid preceded by many months the appearance of bone lesions. These bone lesions are characterized by bone destruction, and also by a limited apposition of new bone. The syndrome observed corresponds closely to the picture of *osteitis fibrosa generalisata*, or Recklinghausen's disease of human pathology. This similarity is underlined by the importance of a time factor in both conditions. Only recently Alexander, Pemberton, Kepler and Broders (1944) have pointed out that not so much severity as the duration of hyperparathyroidism is responsible for the disease in humans.

Our findings are highly suggestive for the assumption that the syndrome described is due to hyperparathyroidism, but we are not yet in a position to explain its ultimate cause. Possibly biochemical studies, now in progress, might shed further light on this problem. At the moment we can only state that the mode of action of the goitrogens on the thyroid must be altogether different from

the one on the parathyroid; thyroid function is regularly affected after a few days, while hyperparathyroidism develops only slowly, and in a small percentage of our animals. The literature on experimental hyperparathyroidism deals mostly with the effect of massive doses of parathormone given for short periods. The changes in the bones in such experiments are similar to the ones described in this paper. However, the importance of the results obtained by Jaffe, Bodansky and Blair (1931) has been questioned by Snapper (1938) and Hunter and Turnbull (1931), because similar changes were found to occur in a great variety of experimental conditions and even spontaneously. Young animals had been used in the majority of such experiments, and in these, osteofibrosis seems easily induced. In our material we have not seen a single animal with osteofibrosis which was younger than 14 months.

Apart from primary hyperparathyroidism (Recklinghausen's disease), enlargement of the parathyroids has been described in a number of conditions. All of these have in common a primary disturbance of the metabolism of calcium and phosphorus (Luce, 1923; Campbell and Turner, 1942), as in rickets and osteomalacia, or they are due to acidosis as in renal failure (Gilmour, 1947). Only in the last condition do bone lesions occur, associated with enlarged parathyroids and resembling Recklinghausen's disease. Pappenheimer (1936) has studied this syndrome in young rats, the renal tissue of which had been greatly reduced. We have no evidence to assume that a kidney disease was the cause of the syndrome described in this paper. Further, it seems necessary to mention that the diets given in our experiments provided an ample supply of both calcium and phosphorus, excluding a nutritional deficiency.

The most controversial issue in parathyroid physiology is the role of the pituitary in regulating the parathyroid function and size. Up to date no conclusive evidence for the existence of a parathyrotropic hormone has been obtained. We have studied the pituitaries of our rats suffering from hyperparathyroidism, and have failed to find any consistent changes in the cells of the anterior lobe. Our material reveals that parathyroid hyperplasia occurred independently of the presence or absence of acidophil cells. There were also no signs indicative for an active secretion of basophils or chromophobe cells. We have, however, observed an enlargement of the pars intermedia, accompanied by certain structural changes, the significance of which is not yet clear. As long ago as 1924 attention to an enlargement of the pars intermedia following parathyroidectomy was drawn by Vines (1924).

SUMMARY.

(1) Long-term administration of goitrogens leads to an enlargement of the parathyroids in the rat.

(2) After 14 months or more of feeding of thiourea or related compounds (thiouracil and methylthiouracil), a generalized osteitis fibrosa was found in 9 of 33 animals in association with enlargement of the parathyroids.

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THYROTROPIC HORMONE IN THYROTOXICOSIS, MALIGNANT EXOPHTHALMOS AND MYXOEDEMA.

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SINCE the discovery of the thyrotropic secretion of the pituitary by Aron, Loeb and Basset (1929), there has been much investigation of its function in controlling the thyroid gland in animals. In particular, it has been demonstrated that the hyperplasia produced by goitrogenic agents is produced by the thyrotropic secretion of the pituitary which can be detected in the blood (Griesbach and Purves, 1943).

However, there has been less success in evaluating the role of this hormone in human clinical syndromes. Hertz and Oastler (1936), using the hypophysectomized rat as a test animal, were unable to demonstrate thyrotropic activity in blood or urine of thyrotoxic patients, although positive results were obtained from myxoedema patients. Cope (1938) was also unable to find any increased thyrotropic hormone in the serum or urine of thyrotoxicosis patients. He used the guinea-pig as a test animal, and showed that the method would detect thyrotropic hormone in the blood of the thyroidectomized rabbit. Galli-Mainini (1942) assayed sera by their metabolism stimulating effects on thyroid tissue slices. He found no increased activity in sera from ordinary thyrotoxic patients, but obtained positive results in cases with dissociated ophthalmopathy.

Nevertheless, it has been assumed by many writers that the syndrome of

thyrotoxicosis is primarily due to an excess of thyrotropic hormone production. It is also now the custom to ascribe the eye signs of the thyrotoxicosis syndrome to the action of excessive amounts of the thyrotropic hormone on the orbital tissues. There are a number of investigations made in animals which tend to show that pituitary extracts having thyrotropic activity do have an effect on the orbital tissues (Smelser, 1937; Dobyns, 1945; Albert, 1945). The hypothesis of over-secretion of thyrotropic hormone in thyrotoxicosis is, therefore, an attractive one, since this single assumption would explain the two otherwise unrelated features of the disease, namely, the excess of thyroid secretion and the ocular signs. However, unless the thyrotropic hormone is found to be present in the blood, it will be necessary to abandon or modify the hypothesis. From the results of our investigations it seems to us that the hypothesis must in fact be abandoned.

No entirely satisfactory method for the assay of human serum for thyrotropic activity has been described. It is necessary to have a sensitive test animal, and also one which can tolerate large injections of serum. We have not considered the possibility of concentrating the hormone from the serum because of the dangers of modifying the activity during the concentration process. Thus Albert, Rawson, Merrill, Riddell and Lennon (1946) described an increase in the activity of inactivated pituitary extracts after heating, either alone, or with reducing substances. Therefore, despite the fact that the chick may be the most sensitive test animal, we have chosen the guinea-pig as the test animal because of its ability to absorb large doses of serum.

In order that the smallest activation of the thyroid may be of significance, it is necessary to treat the guinea-pigs so as to bring their thyroids as far as possible into the completely inactive state. For this purpose we administered thyroid substance which had been brought into solution by peptic digestion, as has been previously described (Griesbach and Purves, 1943). The response of the thyroid to thyrotropic hormone was evaluated by measuring the mean cell height of the acinar epithelium.

MATERIAL AND METHODS.

Litters of guinea-pigs, five to seven days old, were taken and injected with thyroid digest, each daily injection containing 10 μg. of iodine. After nine days of thyroid digest treatment serum injections were begun. The serum was given in the dosage of 2·5 ml. per 100 g. body weight per day, the guinea-pigs being weighed each day for the determination of dosage. The daily dose was given in two injections administered subcutaneously with several hours between injections. In many cases the serum caused a local or general toxic effect, which did not seem to be due to bacterial contamination, but at the dose level used the animals usually survived the course. The serum was given on four successive days. On the fifth day the animals were killed with coal gas and the thyroids dissected out and fixed in 10 per cent formol saline. The sections were cut at 5μ thickness and stained by Heidenhain's Azan method. For the measurement of cell height the sections were projected on to an opaque screen at a magnification of 1000. Cell heights in 50 acini were measured on the screen in mm., each mm. representing one micron. Serum samples were separated from the clot, cleared by centrifuging and preservatized with 0·3 per cent of metacresol.

RESULTS.

The sera examined comprised 22 sera from 19 cases of uncomplicated thyrotoxicosis before treatment, 19 sera from 17 cases of thyrotoxicosis under treatment with thiourea or thiouracil, 37 sera from 27 cases of malignant exophthalmos and 5 sera from 5 cases of myxoedema.

Owing to a shortage of animals and deaths among them during the injections, there has not been a control obtained from every litter of guinea-pigs used for these experiments. However, sufficient controls have been obtained to provide a continuous check on the unstimulated state of the guinea-pig thyroids. The results obtained are summarized in Table I, and the distribution of the values is illustrated by the diagram of Fig. 1.

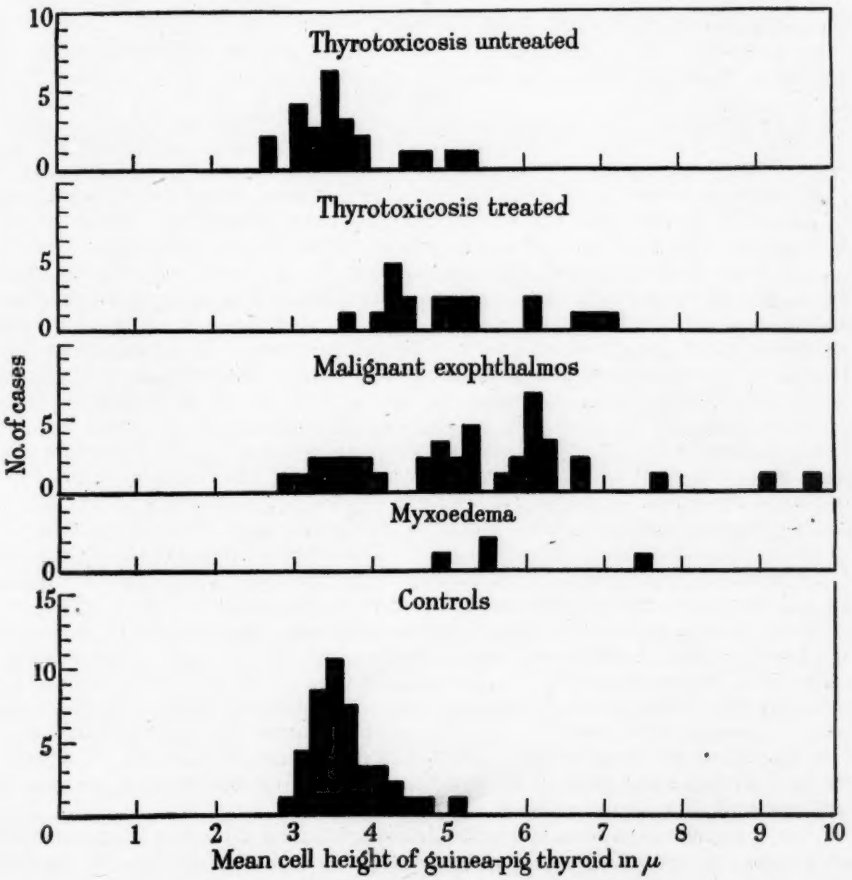

FIG. 1.—Distribution of results of thyrotropin tests.

TABLE I.—*Results of Thyrotropin Tests on Human Sera. Mean Values of Thyroid Cell Heights on Guinea-pigs.*

Sera.	No. of sera examined.	Mean cell height.	Standard error.
Control (no serum)	40	3·58	± 0·07
Thyrotoxicosis untreated	22	3·60	± 0·15
Thyrotoxicosis treated	19	5·09	± 0·24
Malignant Exophthalmos	37	5·21	± 0·24
Myxoedema	5	5·31	± 0·58

TABLE II.

Comparison.	Difference.	Standard error.
Thyrotoxicosis untreated—controls	0·017	± 0·17
Thyrotoxicosis treated—controls	1·51	± 0·25
Malignant Exophthalmos—controls	1·62	± 0·25
Myxoedema—controls	1·73	± 0·58
Thyrotoxicosis treated—untreated	1·50	± 0·27

Comparisons that may usefully be made between these values are shown in Table II. together with the standard errors of the differences. It will be seen that there is no significant difference between the results of guinea-pigs receiving serum from thyrotoxic patients and the control guinea-pigs. On the other hand, the three groups, thyrotoxicosis under treatment with thiourea or its derivatives, malignant exophthalmos, and myxoedema, all show significant increases in cell height as compared with the controls. It is plain, therefore, that thyrotropic activity was present in these sera. However, an examination of the mean value does not present the whole story of the thyrotropic activity of these cases. It is necessary to consider the amount of variation in these groups. For instance, in the group "thyrotoxicosis untreated," the standard deviation of the cell heights was greater than that for the controls (σ for thyrotoxicosis untreated $\pm$ 0·70μ, for controls $\pm$ 0·47μ). An examination of the figures showed that this extra variation in the thyrotoxicosis group was due to three abnormal values for cell height. These three values were 2·6, 2·7 and 5·3. With these three values eliminated, the standard deviation of the remaining 19 values was practically the same as that of the controls. The lowest cell height found in any of the control guinea-pigs was 2·9μ, and the highest 5·0μ. We have considered the possibility of the two low values in the "thyrotoxicosis untreated" group being due to a thyroid depressing activity in these sera, but the evidence is insufficient to support this. The high value, 5·3μ, could be due to the presence of thyrotropic activity in this serum, but the result is not of high statistical significance, and could well be due to chance variation. Therefore, in the thyrotoxicosis group with possible exception of one serum there was no indication of thyrotropic activity.

The standard deviation for thyrotoxicosis treated ($\pm$ 1·03μ) and for malignant exophthalmos ($\pm$ 1·47μ) are significantly greater than that for the controls ($\pm$ 0·47μ). This is to be expected, since thyrotropic activity is present in

varying amounts in the different sera. The standard deviation of the malignant exophthalmos groups is considerably greater than that of the thyrotoxicosis treated group, but this difference is not of great statistical significance (P between 0·1 and 0·05).

An examination of the distribution of the values in the malignant exophthalmos group, as shown in Fig. 1, reveals that while most of the sera tested had definite thyrotropic activity, there was a group of nine sera yielding cell heights between 2·8 and 3·9μ in which thyrotropic activity seems to be definitely absent. It is certain then that thyrotropic activity is not universally present in the blood of patients with malignant exophthalmos.

An examination of the distribution of the values for thyrotoxicosis untreated and after treatment with thiourea or thiouracil as shown in Fig. 1 reveals that the whole group of patients under treatment shows a shift to the right as compared with patients untreated. This indicates that the appearance of thyrotropic activity in thyrotoxic patients under treatment with thiourea or thiouracil is general.

DISCUSSION.

It is of great importance to know for comparative purposes the response to be expected from normal sera. Cope (1938) did not obtain with normal sera any histological activation of the guinea-pig thyroid using a method somewhat similar to our own. We have been able to observe only four specimens of normal human serum, but with these four no activation of the guinea-pig thyroid was detectable as compared with the controls. We have at present in progress a further investigation of the thyrotropic activity of normal sera, but for the purpose of the present investigation we will assume that the effects obtainable from normal sera are negligible when tested by our method.

The results obtained from untreated cases of thyrotoxicosis seem to us to demonstrate conclusively that the hypersecretion and the hyperplastic activation of the thyroid in this disease is not the result of thyrotropic secretion from the pituitary. This result seems most important, since the current literature reveals that many investigators are assuming on insufficient grounds that the hyperthyroid syndrome is due to hypersecretion of pituitary thyrotropin. This hypothesis, which would make the hyperthyroid syndrome a disease primarily of the pituitary or of some mid-brain centre, has strong attractions, since it would seem to offer a hypothesis which could explain both the general activation of the thyroid and the ocular signs of this disease. However, it seems that the results of these tests show that this hypothesis, despite its attractions, must be abandoned. In saying this we are not overlooking the auxiliary hypothesis put forward by Rawson, Graham and Riddell (1943) that the thyrotropin is inactivated by the thyroid in the course of producing its stimulation. It has been postulated that there may be a hypersecretion of thyrotropin which is not detectable, since the thyrotropin is rapidly inactivated by the hyperplastic thyroid. We think that this inactivation could, in fact, account only for the absence of thyroid stimulating action in the blood of the thyroid veins. If the thyroid is being stimulated by the blood which is reaching it from the arteries, then that stimulating action should be demonstrable in blood drawn from the general circulation and should reveal itself in the guinea-pig. The inactivation of thyrotropic hormone by the thyroid may result in a lower level of thyrotropic

activity in the blood than would be attained after thyroidectomy, but it should still be detectable.

The results of the test of sera from thyrotoxic patients after treatment with thiourea or thiouracil show that thyrotropic activity appears in the blood during treatment. This thyrotropic activity in the blood of thyrotoxic patients under treatment seems to us to show that pituitary function is substantially normal in hyperthyroidism. Thus, while thyrotropic activity is absent while the thyroxine level of the blood is high, there is a secretion of thyrotropin when the thyroxine level has been reduced to normal or slight below normal levels. However, it could be claimed that the appearance of thyrotropic activity during treatment with thiourea or its derivatives is due to the reactivation of inactivated thyrotropic hormone by the action of the thiourea (Albert, Rawson, Merrill, Lennon and Riddell, 1947). The observations of Wegelin (1938) on the histology of the pituitary in 20 cases of hyperthyroidism are most important in this connection and seem to us to be crucial. He found the histology of the pituitary in hyperthyroidism to be substantially normal with no signs of hyperfunction. Since secretion of thyrotropin is associated with hypertrophy and hyperplasia of basophil cells which are easily recognized, the absence of such changes in hyperthyroidism indicated that there is no excess secretion of thyrotropin in this disease.

Although the thyrotropin hormone is not involved in the production of the hypersecretion of the thyroid and the eye signs of ordinary thyrotoxicosis, it could still be responsible for the severer orbital changes of malignant exophthalmos. The hypothesis that excessive thyrotropin secretion has a causal role in the production of malignant exophthalmos obtains its main support from the demonstration of the exophthalmos-producing effects of pituitary extracts in laboratory animals. There has been, however, little attempt to demonstrate the existence of this excessive thyrotropin in the blood of such patients. Our own tests show that a major proportion of such malignant exophthalmos cases do, in fact, have high amounts of thyrotropin in the serum. Nevertheless, the existence of typical cases of this syndrome with a complete absence of thyrotropic activity in the serum raises important doubts concerning the causal role of thyrotropin in this syndrome. The group of cases without thyrotropic activity in the serum did not differ in any clinical feature from the group with elevated thyrotropic activity. Early cases and very severe cases appeared in both groups. Moreover, high levels of thyrotropic hormone were found in myxoedema patients and in thyrotoxicosis patients after thiourea or thiouracil treatment.

The infrequent occurrence of malignant exophthalmos compared with the numbers of thyrotoxic patients being treated shows that in most individuals the presence of excess thyrotropic hormone in the blood does not lead to malignant exophthalmos. Orbital changes are also certainly not a marked feature of myxoedema. It therefore appears that a high level of thyrotropic hormone does not, in general, produce a malignant exophthalmos, nor is a high level invariably found in malignant exophthalmos patients. It should be noted that in some of the malignant exophthalmos patients in which no thyrotropic hormone was found, the serum was taken in the early stages of the disease when the symptoms were increasing rapidly. Therefore, it cannot be said that the absence of thyrotropic hormone was due to the subsidence of the thyrotropic hormone

after a period during which it had been high and had produced irreversible changes in the orbit. The conclusion that we reach is that there is a considerable degree of correlation between malignant exophthalmos and high serum thyrotropin, but there is not apparently a direct causal relation between the two. Either malignant exophthalmos must be divided into two or more diseases, in only one of which thyrotropic hormone is a causal agent, and even then requires the existence of some other circumstances to produce symptoms of the severity of malignant exophthalmos, or the thyrotropic hormone and the malignant exophthalmos are only indirectly related. In the latter case we would postulate that both the exophthalmos and the hypersecretion of thyrotropin are the results of some primary lesion, presumably in the central nervous system.

When we began this investigation we were inclined to the view that malignant exophthalmos and thyrotoxicosis were two aspects of the same disease. In both syndromes hyperthyroidism and orbital changes may be observed. In malignant exophthalmos orbital symptoms overshadow the metabolism disturbance, which may indeed be absent. On this view the condition which is here called malignant exophthalmos has been termed Graves' disease with disassociated ophthalmopathy. We expected that the severe eye symptoms of malignant exophthalmos would be found to have the same cause as the less severe eye symptoms associated with typical Graves' disease. The fact that so many of the malignant exophthalmos patients had high thyrotropic activity in the blood which was never found in the typical hyperthyroidism makes it likely that the two diseases are distinct.

SUMMARY.

A method for the assay of thyrotropic hormone in serum from human subjects is described.

The results of the examination of 22 specimens from cases of thyrotoxicosis before treatment, 19 specimens from cases of thyrotoxicosis under treatment, 37 specimens from cases of malignant exophthalmos, 5 specimens from cases of myxoedema are described.

In thyrotoxicosis untreated no thyrotropic hormone was found.

In treated thyrotoxicosis weak or moderate thyrotropic activity was present in all cases.

Malignant exophthalmos cases fell into two groups. One group was devoid of thyrotropic hormone. In the other group definite thyrotropic activity was present, in some cases extreme activity being found.

Marked thyrotropic activity was found in untreated myxoedema.

It is concluded that thyrotoxicosis is not the result of excessive secretion of thyrotropic hormone.

The appearance of thyrotropic activity in thyrotoxic patients after treatment is held to show that pituitary function is substantially normal in thyrotoxicosis.

The inconsistency of appearance of thyrotropic hormone in malignant exophthalmos cases is considered to disprove the causal role of thyrotropic hormone in the production of malignant exophthalmos.

It is considered that the excess thyrotropin secretion and the eye lesions of malignant exophthalmos are the result of a lesion in the brain-stem which affects the orbit and the pituitary by neural pathways.

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THE EFFECT OF ACRIDINE COMPOUNDS ON MITOSIS AND OXYGEN CONSUMPTION IN THE ISOLATED BRAIN OF THE EMBRYONIC CHICK.

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THE effect of mitotic poisons on the metabolism of isolated tissues is of importance both as a means of selecting those substances with a high specificity towards cells in division, and as a means of investigating their mode of action. In this respect the effect on oxygen consumption has a special importance, because of the conclusion of Fisher and his colleagues (Fisher and Henry, 1944) that inhibition of cell division is associated with the inhibition of a fraction of the total cellular respiration concerned in this process.

Recently a series of acridine compounds has been shown to inhibit mitosis in cultures of chick fibroblasts, and has been made available through the kindness of Dr. J. H. Wilkinson. The effect of four of these compounds, both on mitosis and on respiration, has been tested on the isolated brain of the embryonic chick.

MATERIAL AND METHODS.

The tissue used was the mid-brain vesicle of the 6-day chick removed from the embryo with its mesodermal and ectodermal coverings. At this stage there are many dividing cells in the ependymal layer which can be readily examined by the aceto-lacmoid squash method of La Cour (Darlington and La Cour, 1942). Although accurate mitotic counts are not possible, about 5 per cent of the cells in the preparations were in some stage of mitosis.

Oxygen consumption was measured by a modification of the Cartesian diver micromanometer previously described (O'Connor, 1948). This technique was

designed to measure the oxygen consumption of *Daphnia*, and certain further modifications have been necessary. The medium was a strongly buffered Tyrode solution of the following composition:

Tyrode solution . .	100 ml.
Na_2HPO_4 2·11 per cent .	24 ml.
KH_2PO_4 2·04 per cent .	6 ml.

the solution being isotonic and of pH 7·6. The amount of buffer was considered an advantage since, in the divers, the tissue was suspended in a small amount of fluid. When removed from the embryo the brain tissue was placed in this medium, and cut into fragments about 1 mm. square. Divers filled with medium were placed beneath the surface of the medium, and four or five fragments introduced into the necks of the divers. The divers were then centrifuged to make the tissue enter the bulb. Excess medium was removed, leaving the tissue fragments suspended in 20–30 μl. of fluid. Subsequent steps of filling the divers followed the technique already described, and included the introduction into the necks of the divers of a solution of the acridine compound to be tested. In most cases the solvent was the medium, but in certain cases limits of solubility made it necessary to use normal saline. In these cases the alteration of the saline composition of the bulb contents resulting from the addition of the acridine solution affected neither mitosis nor respiration. All experiments were performed at 37° C., and the divers were filled with oxygen.

The normal rate of respiration was measured for 45 minutes and recorded as the change in the flotation pressure. The actual amount of oxygen consumed was not calculated, but the change in flotation pressure was 100–150 mm. water, and corresponded to an oxygen uptake of approximately 1 μl. At the end of 45 minutes the acridine solution was added to the bulb contents, and any alteration in the rate of oxygen consumption measured. In order to allow for the mixing of the added acridine solution, the change in flotation pressure was recorded during the period 40–120 minutes after its addition, and the percentage alteration in oxygen consumption calculated. Control experiments were performed in which medium containing no acridine compound was added to the bulb contents from the neck of the divers. In twenty such experiments the oxygen consumption following this manoeuvre averaged 96·0 per cent of the normal, with a standard deviation of 5·08 per cent, so that an alteration of oxygen consumption of less than 10 per cent could not be considered significant.

After two hours' exposure to the acridine compound the tissue was washed from the divers and examined by the aceto-lacmoid squash method. In control experiments mitotic figures showed no abnormality (Fig. 1), but with effective concentrations of acridine compounds recognizable changes occurred. The slightest change observed consisted of swelling and irregularity of chromosomes, which at slightly higher concentrations became fused to a pycnotic mass (Fig. 2), the course of events resembling that described by Dustin (1947). Since it was easier to recognize the fusion to a pycnotic mass, the concentration of acridine compound producing this change was recorded as the "effective mitotic concentration." Although this was a convenient method of comparing the acridine compounds investigated, it is desirable to point out that the results may not be comparable with methods using mitotic counts and where the compound is applied for longer periods of time.

EXPERIMENTAL RESULTS.

(a) *Effective mitotic concentration.*

This concentration in the case of 5-amino-acridine hydrochloride, 5-amino-10-methylacridinium bromide, 5-amino-1-phenyl-10-methylacridinium bromide, and 5-amino-2-trifluoromethylacridine hydrochloride is recorded in Table I.

TABLE I.—*The Effect of Acridine Compounds on Mitosis and on Respiration in the Isolated Brain of the 6-day Chick Embryo.*

Compound.	Effective mitotic concentration mg./100 ml.	Respiratory inhibition— at effective mitotic concentration. %	at 10× effective mitotic concentration. %	at 16 mg./100 ml. %
5-amino-1-phenyl-10-methylacridinium bromide	1·8	0	0	0
5-amino-10-methylacridinium bromide	0·7	0	0	0
5-amino-acridine hydrochloride	0·8	< 10	20	30
5-amino-2-trifluoromethylacridine hydrochloride	0·9	< 10	68	100

The results are averages from a series of experiments as described above, and from those where the effective mitotic concentration was separately tested. In these cases fragments of the brain tissue were exposed to varying concentrations of the acridine compounds in small test-tubes filled with oxygen, and adequate oxygenation assured by reducing the amount of medium to a layer 2–3 mm. in depth. The results in these two types of experiment did not differ. Most experiments involved decreasing concentrations by two-fold dilutions, and there was a subjective element in determining the effective mitotic concentration. Hence it is necessary to suggest that, for two results to differ significantly, one should be twice the other. From Table I it is seen that 5-amino-1-phenyl-10-methylacridinium bromide is less effective than the other three compounds, which show no significant difference.

(b) *Effect on respiration.*

The same four compounds as in the previous section were tested. In each case 20–30 varying concentrations were used, and the relationship of concentration to inhibition of oxygen consumption plotted in Fig. 3, where the concentration is expressed on a logarithmic scale. Certain relevant information is extracted from the figure and included in Table I. Thus it is seen that, at the effective mitotic concentration, the inhibition of respiration is small, none being demonstrated in the case of 5-amino-10-methylacridinium bromide and 5-amino-1-phenyl-10-methylacridinium bromide, while in the case of 5-amino-acridine hydrochloride and 5-amino-2-trifluoroacridine hydrochloride the decrease is less than 10 per cent, and thus not certainly significant. At ten times the effective mitotic concentration there is, however, a considerable difference, there being no demonstrable inhibition in the case of 5-amino-10-methylacridinium bromide and 5-amino-1-phenyl-10-methylacridinium bromide, while 5-amino-acridine hydrochloride and 5-amino-2-trifluoromethylacridine hydrochloride produce inhibitions of 20 per cent and 68 per cent respectively.

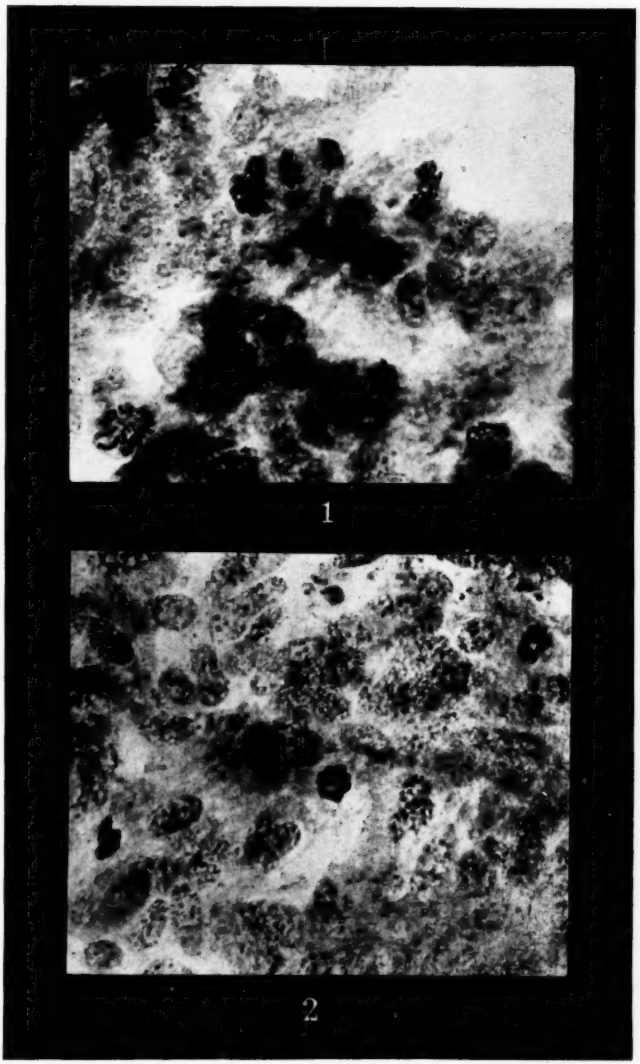

Fig. 1.—Normal mitotic figures.

Fig. 2.—Mitotic figures after 2 hr. exposure to an effective concentration of an acridine compound.

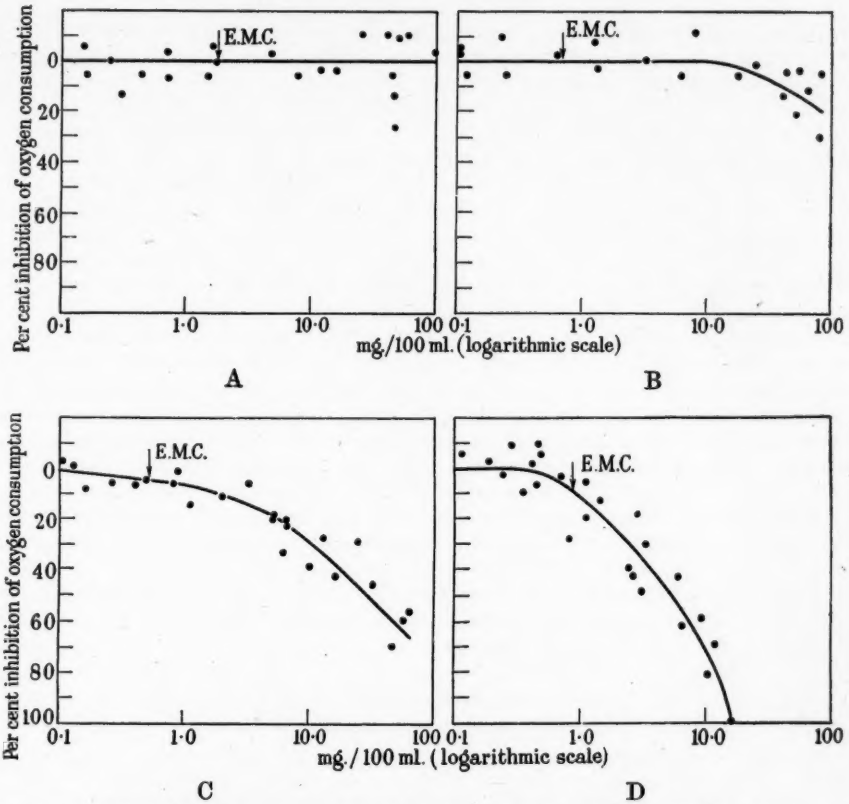

FIG. 3.—The effect of acridine compounds on respiration. E.M.C. = effective mitotic concentration.
A. Concentration of 5-amino-1-phenyl-10 methylacridinium bromide.
B. Concentration of 5-amino-10-methylacridinium bromide.
C. Concentration of 5-amino-acridine hydrochloride.
D. Concentration of 5-amino-2-trifluoromethylacridine hydrochloride.

DISCUSSION.

Two of the compounds, 5-amino-acridine hydrochloride and 5-amino-2-trifluoromethylacridine hydrochloride, produce an increasing inhibition of respiration as the effective mitotic concentration is exceeded. Therefore, if these two compounds were used in intact animals, it is more likely that they would effect general cellular metabolism than the other two compounds.

The inhibition of respiration cannot be due to effects confined to cells in division, since these comprise about 5 per cent of the total cells. It must therefore be due to an inhibition of the respiration of all cells. In the case of 5-amino-10-methylacridinium bromide, 5-amino-acridine hydrochloride, and 5-amino-2-trifluoromethylacridinium bromide hydrochloride, where the effective mitotic concentration is approximately equal, the inhibition of respiration, at equal concentrations, ranged from *nil* to 100 per cent (Table I). It is therefore

concluded that the effect on mitosis and the effect on respiration are independent. A comparable independence has been recorded with similar acridine compounds between antimalarial activity and the inhibition of the respiration of chick red cells infected with *Plasmodium gallinaceum* (Albert and Marshall, 1948).

It is of interest to compare these results with those of Fisher and his co-workers, who investigated the effect of urethane on yeast (Fisher and Stern, 1942), on *Tetrahymena* (Ormsbee and Fisher, 1944), and of both urethane and chloretone on the egg of *Arbacia* (Fisher and Henry, 1944). They concluded that total cell respiration could be divided into two fractions, one, the "active" respiration associated with cell division, and the other, the "resting" associated with other cellular processes. They further concluded that the active respiration was more sensitive to the action of urethane and chloretone than the resting, and that inhibition of cell division was associated with the inhibition of the active respiration. This, in different material, amounted to 20–40 per cent of the total respiration.

In the present series of experiments with acridine compounds no similar decrease of respiration at the effective mitotic concentration has been demonstrated. This difference may, however, be only apparent because of the lower rate of cell division in the material employed, so that any associated oxygen consumption might be so small that its inhibition could not be detected by the method used. Therefore, it is not excluded that the action of acridine compounds on mitosis is associated with the inhibition of an associated fraction of the total oxygen consumption, which, when compared with the remainder, shows a greater susceptibility to the action of acridine compounds, and a smaller susceptibility to variations in their chemical structure.

SUMMARY.

(1) Simultaneous measurements have been made of the effect of four acridine compounds on mitosis and on respiration in isolated brain of the 6-day chick embryo.

(2) All four compounds affected dividing cells in concentrations ranging from 0·7–1·8 mg. per 100 ml., with little or no inhibition of respiration. At higher and equal concentrations the inhibition of respiration ranged from *nil* to 100 per cent.

(3) It is concluded that the effects on mitosis and on respiration are independent.

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RIFT VALLEY FEVER; TRANSMISSION OF THE VIRUS BY MOSQUITOES.

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A CONSIDERABLE amount of evidence was found by Daubney and Hudson (1931, 1933) which indicated that the virus of Rift Valley fever is transmitted by mosquitoes. Nevertheless, it was not until 1944 that the agent was first isolated from wild-caught mosquitoes (Smithburn, Haddow and Gillett, 1948); and prior to the present communication there has been no account of its successful transmission by hematophagous arthropods.

In 1944, while attempting to discover the vector responsible for the transmission of yellow fever among wild primates in uninhabited forest (Smithburn, Haddow and Gillett), Rift Valley fever virus was isolated 6 times from different lots of mosquitoes caught during a period of 39 days in a circumscribed area of the Semliki Forest in western Uganda. The agent was isolated once from *Aëdes* (*Stegomyia*) *de-boeri* spp. *de-meilloni*, Edwards, twice from the *Aëdes* (*Aëdimorphus*) *tarsalis*, Newst. group, and 3 times in successive catches from *Eretmapodites* spp. The indications were that the former 2 contained little virus and may have been only casually infected, whereas the *Eretmapodites* not only were regularly infected during the period of virus activity, but apparently contained the virus in considerable quantity. These facts suggested that the local vector, if one was included in the species from which virus was isolated, was one of the *Eretmapodites* spp. Mosquitoes of the *Eretmapodites chrysogaster* Graham group were more numerous in the infected lots than were any of the other species of Eretmapodites. Moreover, members of this group were known to be capable of transmitting another virus disease—yellow fever (Bauer, 1928). Accordingly, it was decided to attempt the experimental transmission of Rift Valley fever virus with these mosquitoes, and with other species regarded as suspect vectors of the virus in Kenya. The tests with *Eretmapodites* were successful, and are described in this paper, which therefore records the first experimental transmission of Rift Valley fever virus by the bites of mosquitoes.

MATERIALS AND METHODS.

At the time these experiments were carried out we had no colony of mosquitoes of the *E. chrysogaster* Graham group, and we therefore were obliged to use wild insects, or adults reared from wild-caught larvae. These were captured at Kitinda, a forested locality near Entebbe, where they were present in good numbers, and where Rift Valley fever was not known to have occurred. Adult female mosquitoes were caught individually in glass tubes as they alighted to bite the catchers, and the tubes were then lightly plugged with cotton. The captured insects were transported the short distance to the laboratory in the

catching tubes ; there they were examined and sorted by one of us and released into Barraud cages.

The *Taeniorhynchus* employed in the experiments were also wild mosquitoes, which were caught in lake-shore localities near this Institute. The *Aëdes aegypti* were laboratory-reared insects descended through several generations from material collected in Nigeria by Dr. J. C. Bugher, who supplied us with the mosquito eggs.

The animals used were mice and lambs. The lambs were European-native hybrid stock and were about 5 or 6 months old. None of them died as result of Rift Valley fever in these or other experiments. Whether their survival was due to age, or breed, or to characteristics of the virus is not known. Their clinical and immunological responses to infection, whether induced by bites of mosquitoes or by inoculation, were typical.

The mosquitoes received their infective feeds, in the first instance, from mice sick or moribund following inoculation with virus of Rift Valley fever. The inoculated mice were placed singly in close-fitting cylinders made of monel-metal gauze. Each end of the cylinder was closed with a cork to render the mouse relatively immobile without causing it discomfort. One of the corks was grooved to accommodate the animal's tail. In some instances moribund mice were exposed to mosquitoes without being confined in the cylinders. The infected mice in the cylinders (and the few not so confined) were placed in Barraud cages.

The mosquitoes which gorged were removed to other Barraud cages. All those which fed on a given day on mice of the same group were placed in the same cage and given the same lot number. The cages were kept in a controlled temperature cabinet. The cages and the floor of the cabinet were covered with moist cotton in order to provide a highly humid atmosphere for the insects. The temperature within the cabinet was 30° C. throughout the experiments. Mosquitoes of the genus *Taeniorhynchus* did not thrive well in the controlled temperature cabinet and, after a few preliminary trials, they were kept in large Barraud cages in the open air of the insectary, with only the tops of the cages covered with moist cotton. The temperature of the insectary varied between 22° C. and 26·6° C. during the experiments. The mosquitoes were given banana and water daily, but on the day before they were to be exposed to normal mice or a normal lamb for transmission attempts, the banana was removed from the cages.

The mosquitoes to be offered a transmission feed on a lamb were placed individually in wide-mouthed gauze-covered glass tubes, which were applied to the shaven skin of the animal. The transmission feeds on mice were given in the same manner as the infective feeds.

When the first transmission of the virus to a lamb was accomplished, fresh lots of mosquitoes were exposed to this animal. Those which gorged were thereafter handled in the same way as mosquitoes which received their infective feeds from mice.

An animal which served as source of virus for the mosquitoes was usually bled from the heart in the case of a mouse (for this and other procedures the animals were anaesthesized with ether), or the jugular vein in the case of a lamb, immediately after the infective feed, and the serum was tested for virus. In the case of the lamb sera, titrations of virus content were done.

During the experiments infected mosquitoes were inspected daily, in the early part of the morning. Any which were found dead were ground up, usually individually, for inoculation into mice to determine whether they had retained the virus. For these tests, mosquitoes were triturated in a mortar with 1 ml. of 10 per cent non-immune serum in physiological saline (hereafter referred to as serum-saline or diluent). The suspension was spun in an angle centrifuge at about 2500 r.p.m. for 15 to 30 minutes. A group of mice was inoculated intraperitoneally, with 0·06 ml. of the supernate per mouse, after which the remainder of the supernate was passed through a Seitz E.K. pad previously washed with serum-saline. A second group of mice was inoculated intraperitoneally with this Seitz filtrate.

When a lamb bitten by infected mosquitoes exhibited an elevation of temperature it was bled, and its serum was tested for circulating virus. When quantitative virus determinations were required, end-points were calculated by the method of Reed and Muench (1938), and the end-point dilution was regarded as containing 1 LD_{50} per unit of inoculum.

The livers of mice which sickened or died as the result of being bitten by infected mosquitoes were employed for passage to other groups of mice. Inoculated mice were examined once daily, or oftener when necessary. Protection tests were made to identify virus recovered from mosquitoes. The identity of virus recovered from mosquitoes was further confirmed by histological examination of livers of mice inoculated with suspensions of the mosquitoes.

The protection tests were done in the following manner: 0·25 ml. portions of material suspected of containing Rift Valley fever virus (lamb serum, or the unfiltered supernate of a 10 per cent suspension of liver from a sick or a dead mouse) were added to 0·5 ml. portions of known non-immune serum and to specific Rift Valley fever immune serum. After vigorous agitation the serum-virus mixtures were allowed to stand for 15 minutes to 1 hour, at either room or incubator temperature, after which separate groups of 6 mice were inoculated intraperitoneally with 0·06 ml. of the mixtures per mouse. The presence of Rift Valley fever virus was considered proved if not more than 1 mouse receiving the mixture containing non-immune serum survived and if all, or all but one, of those receiving the mixture containing Rift Valley fever immune serum remained well during a period of 10 days following inoculation. Tests for immunity were made in convalescent lambs by mixing stock virus with the serum of the lambs in question. This was incubated and inoculated in the same manner as the serum-virus mixtures for protection tests. Suitable controls were employed.

The macroscopic appearance of the livers of infected mice was found to be so variable as to be unreliable for diagnostic purposes, but the presence of Rift Valley fever could easily be detected by microscopic examination of this tissue. In no instance was there disagreement between the results of protection tests and the histological examination.

EXPERIMENTAL.

Eretmapodites chrysogaster Graham group.

Four lots of mosquitoes of the *E. chrysogaster* Graham group were found to, or appeared to, have received their infective feeds at times when the level of circulating virus was adequate in the infecting animal, and 3 of these lots

gave successful transmissions, as will be seen from the following paragraphs. Table I shows the number of mosquitoes of each lot which bit normal animals on various days, and indicates those bitings which resulted in transmissions.

TABLE I.—*Mosquitoes of the Better Infected Lots of each Species, showing Number which Probed or Gorged on Normal Animals at Various Intervals after their Infective Feeds.*

Days after infective feeds.	No. mosquitoes which probed or gorged on mice.							No. mosquitoes which probed or gorged on lambs.						
	Aëdes aegypti.	*Eretmapodites chrysogaster* group.				*Taeniorhynchus fuscopennatus.*		*Aëdes aegypti.*	*Eretmapodites chrysogaster* group.				*Taeniorhynchus fuscopennatus.*	
	Lot 5	Lot 1	Lot 3	Lot 7	Lot 11	Lot 15	Lot 17	Lot 5	Lot 1	Lot 3	Lot 7	Lot 11	Lot 15	Lot 17
3	..	..	..	..	13	..	..	..	..	..	..	..	..	..
5	4	..	..	..	Many	..	..	..	..	..	..	..	..	..
6	..	..	..	3	..	..	2	..	..	..	..	..	..	..
7	7	..	..	..	11	..	..	..	..	..	..	..	..	..
8	..	..	..	..	..	..	Several	..	..	..	..	..	..	..
9	..	..	..	..	Several	..	..	..	..	..	..	..	..	..
10	..	..	2	..	..	..	4	7	..	..	4	..	..	..
11	..	..	..	1	5	..	..	..	2	..	..	..	..	..
12	1	..	..	..	..	2	3	..	..	..	..	..	..	..
13	..	..	..	..	4	..	2	..	..	1	4†	..	..	..
14	..	..	..	..	..	..	..	5	..	..	..	..	..	..
15	..	..	..	..	3	..	..	..	..	..	..	..	..	..
16	..	..	..	..	..	..	..	..	..	..	..	..	1	..
17	..	..	..	..	..	..	..	..	..	..	..	7	..	1
18	..	..	..	..	..	..	..	5	..	..	..	..	..	..
19	..	..	..	..	2*	..	..	..	..	..	..	..	..	..
20	..	..	..	4*	..	..	..	..	3*†	..	..	3*.	1	10
21	..	..	..	..	..	..	..	1	..	..	..	..	..	..
22	..	..	..	..	1	..	..	..	..	..	..	2	..	6
23	..	..	..	..	..	..	..	..	..	..	..	..	1	..
24	..	..	1	..	..	..	..	2	..	..	..	..	..	4
25	..	..	..	..	1	..	..	..	..	..	..	1	1	..
27	..	..	..	..	..	..	..	..	..	..	..	..	1	5
29	..	..	..	..	..	..	..	..	..	..	..	1	..	3
31	..	..	..	..	..	..	..	..	..	..	..	1	..	1
32	..	..	..	..	..	..	..	..	..	..	..	..	1	..

* Transmission occurred.

† Mosquitoes of Lots 1 and 7, which received their infective feeds 6 days apart, bit Lamb 3 on the same day. It is probable that the transmission was effected by Lot 1.

Transmission 1.—Twelve *E. chrysogaster* group mosquitoes, comprising Lot 1, received their infective feeds from mice on May 12. Although they were afterward repeatedly offered feedings on normal mice in attempts to secure transmission of the virus, none of them bit these animals. Two of the lot bit normal Lamb 3 on the 11th day after their infection without results. Three of the lot bit the same animal on the 20th day after their infection (June 1), together with 4 *E. chrysogaster* group mosquitoes of Lot 7, which had received their infective feeds on May 18, 6 days after the mosquitoes of Lot 1 (Table I). On June 4 the lamb's temperature rose sharply to 107·2° F. (Fig. 1), and remained elevated throughout the day, but it was within normal limits by the following morning.

Six tests for virus were made with mosquitoes of Lot 1 from the 15th to the 26th day after their infective feeds. Five of the tests were made with individual mosquitoes, and the 6th with a pool of 2. The pool of 2 mosquitoes and 3 of

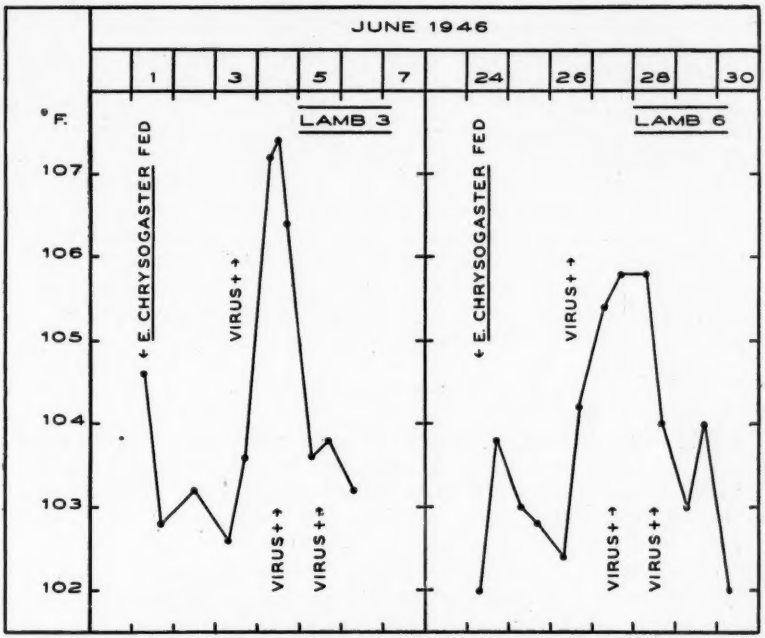

Fig. 1.—Temperature charts of Lamb 3 and Lamb 6.

the individual mosquitoes contained virus, so that either 4 or 5 of the 7 mosquitoes tested were shown to have retained the virus, all of them for more than 2 weeks.

Lamb 3 was bled daily for 10 days, and groups of mice were inoculated intraperitoneally with its serum to test this for the presence of circulating virus. Serum specimens taken on June 4 (the day of fever) and on June 5 were titrated, while on other days only the undiluted serum was tested. Sufficient virus was present in serum taken June 3 to cause the death of all the inoculated mice. On June 4 the titre of virus in the serum of the lamb was 1 in 34,000, and on June 5 it was 1 in 69 (Table II). No virus was present in blood taken on other days.

A protection test employing as virus the serum taken from Lamb 3 on June 4 against normal and Rift Valley fever immune sera, showed that the infective agent present that day in the serum of the lamb was specifically neutralized by the Rift Valley fever immune serum (Table II). Additional protection tests, employing preinfection serum, and specimens taken at various stages in the convalescence of the lamb, against stock Rift Valley fever virus, showed that demonstrable protective antibody was present 7 days after the onset of fever, or 10 days after the animal was bitten by infected mosquitoes (Table III).

Histological studies were made of the liver of a mouse inoculated with serum taken from Lamb 3 on June 3. These revealed the presence of the characteristic lesions of Rift Valley fever, in further confirmation of the transmission.

TABLE II.—*Results of the Intraperitoneal Titration of the June 4 Serum of Lamb 3, and of a Protection Test which showed that the Pathogenic Agent in that Serum was Rift Valley Fever Virus.*

Serum.	June 4 serum, Lamb 3, dilution, log.	Deaths of mice (days after inoculation).	Summary of result. Died.	Summary of result. Lived.	Titre of virus, 1 in—
Normal rhesus	0	2, 2, 2, 3, 3, 3	6	0	..
„ „	2	3, 3, 3, 3, 3, 5	6	0	..
Immune human	0	S, S, S, S, S, S	0	6	..
„ „	2	S, S, S, S, S, S	0	6	..
Diluent only, for titration of virus in serum of Lamb 3	0	2, 2, 2, 3, 3, 3	6	0	34,000
	1	3, 3, 3, 3, 3, 4	6	0	
	2	2, 3, 3, 3, 3, 3	6	0	
	3	3, 3, 3, 3, 3, S	5	1	
	4	3, 3, 3, 3, 3, S	5	1	
	5	3, 3, S, S, S, S	2	4	
	6	S, S, S, S, S, S	0	6	

S indicates that the mouse survived.

It could not be determined whether Lamb 3 was infected by mosquitoes of Lot 1 or Lot 7. However, in the light of results which follow, it seems probable that it was infected by the mosquitoes of Lot 1, and that the extrinsic incubation period was 20 days.

TABLE III.—*Results of Two Intraperitoneal Mouse Protection Tests employing 1 per cent Rift Valley Fever Virus, Showing the Acquisition of Humoral Immunity by Lambs Infected through the Bites of* Eretmapodites chrysogaster *Group.*

Serum.	Days after infecting bites.	Deaths of mice (days after inoculation).	Summary of result. Died.	Summary of result. Lived.
Lamb 3, preinfection	..	2, 2, 2, 2, 3, 4	6	0
June 7	6	3, 3, 3, 3, 3, 3	6	0
„ 8	7	3, 3, 3, 3, 3, 3	6	0
„ 9	8	3, 3, 3, 3, 4, 4	6	0
„ 10	9	5, 5, 6, 6, S, S	4	2
„ 11	10	4, S, S, S, S, S	1	5
„ 17	16	S, S, S, S, S, S	0	6
Lamb 4, normal	..	2, 2, 2, 2, 3, 3	6	0
5, „	..	2, 2, 2, 2, 3, 3	6	0
1, immune	..	S, S, S, S, S, S	0	6
Lamb 4, normal	..	2, 2, 2, 2, 2, 2	6	0
5, „	..	2, 2, 2, 2, 2, 5	6	0
6, preinfection	..	2, 2, 3, 3, 3, 3	6	0
6, July 5	11	S, S, S, S, S, S	0	6
1, immune	..	S, S, S, S, S, S	0	6

S indicates that the mouse survived.

Transmission 2.—The 33 *E. chrysogaster* mosquitoes comprising Lot 7 received infective feeds from mice on May 18. One gorged on and 2 probed a normal mouse on the 6th day after the infective meal, and another probed a normal mouse on the 11th day, but no transmission occurred. Four of the mosquitoes gorged on a normal lamb on the 10th day, without transmitting virus; another 4 bit a normal lamb (No. 3) on the 14th day, probably with negative result. The final exposure to normal animals occurred on the 20th

day (June 7), when 4 of the insects bit a normal mouse (Table I). The mouse remained well during 48 hours, but was found dead on the morning of the 3rd day. It was inadvertently discarded before a protection test was made or the liver was obtained for histological examination. However, the death of the animal within the appropriate period of time and after an illness of less than 24 hours is indicative of Rift Valley fever. Moreover, all the mosquitoes that bit this mouse were shown to be infected. On the 21st day after the infective feeds the 13 mosquitoes remaining alive, including the 4 which bit a normal mouse the previous day, were tested individually for the presence of virus. Each of the 13 contained virus. Sixteen other tests for virus were made on individual mosquitoes of this lot between the 8th and the 20th day after the infective meals ; 14 gave positive results. The tests on this lot, therefore, showed that 27 of 29 mosquitoes tested had retained the virus, 13 of them for as long as 21 days. The extrinsic period of incubation in this transmission was 20 days, and the intrinsic period was 3 days.

Transmissions 3 *and* 4.—The 44 *E. chrysogaster* group mosquitoes of Lot 11 received their infective feeds on Lamb 3 on June 4, at a time when the serum of the lamb contained 567,800 LD_{50} of virus per ml. The lamb itself had been infected by *E. chrysogaster* group mosquitoes. Mosquitoes of Lot 11 bit normal mice on the 3rd, 5th, 7th, 9th, 11th, 13th and 15th days after their infective meals without transmitting virus. A normal mouse bitten by 2 of the mosquitoes on the 19th day (June 23) became infected (transmission 3). Individual mosquitoes probed normal mice on the 22nd and 25th days, but got no visible blood, and did not transmit virus. Five gorged on and 2 probed non-immune Lamb 4 on the 17th day, but transmission did not occur. One gorged on and 2 probed normal Lamb 6 (Table I) on the 20th day (June 24), and this lamb became infected (transmission 4). Two mosquitoes probed normal Lamb 4 on the 22nd day and one on the 25th day, but got no blood and did not infect that lamb. One mosquito gorged on normal Lamb 4 on the 29th day and again on the same lamb on the 31st day without result. This mosquito was tested on the 31st day and found to contain virus. Its failure to transmit is unexplained, but probably indicates that the vector potential of mosquitoes of the *E. chrysogaster* group is variable. Forty-two tests of individual mosquitoes of this lot showed that 33 of them retained virus.

In summary, it may be said that transmission from lamb to lamb and from lamb to mouse was effected with mosquito Lot 11, with extrinsic incubation periods of 20 and 19 days respectively, and that 78 per cent of the mosquitoes tested were proved by inoculation to have retained the virus, the longest period of retention being 31 days.

The normal mouse bitten by mosquitoes of this lot on June 23 (transmission 3) remained well during 4 days, but was sick on the morning of the 5th day. It was sacrificed, and a suspension of a portion of its liver was used as virus in a Rift Valley fever protection test. Of the 6 mice which received this suspension mixed with non-immune serum, 4 were dead on the 2nd and 2 on the 3rd day. Each of the 6 mice receiving the liver suspension mixed with Rift Valley fever immune serum remained well during 10 days. The result showed that the liver of the mouse contained an infective agent which was specifically neutralized by antibody against Rift Valley fever virus. The transmission was further confirmed by the fact that the liver of the mouse infected by the mosquitoes of

Group 11 exhibited specific lesions of Rift Valley fever. The incubation periods in this transmission were: extrinsic, 19 days; intrinsic, 5 days.

Lamb 6, bitten by mosquitoes of Lot 11 on June 24 (Transmission 4), remained afebrile and appeared well until the afternoon of June 26, when its temperature was slightly elevated and it seemed listless. The temperature was further elevated the next day (Fig. 1), and did not return to normal until June 29th. Tests in mice of serum taken from Lamb 6 showed that virus was present in its blood on June 26, 27, and 28, but not on other days. Quantitative tests were done on June 27 and 28, and titres of virus in the serum were 1 in 15,800 and 1 in 372 respectively. The liver of a mouse ill as result of inoculation with the serum of Lamb 6 taken on June 26 was used (as virus) in a Rift Valley fever protection test. Five of 6 mice receiving the suspension of this liver mixed with normal serum succumbed on the 2nd and 3rd days, while 5 of 6 receiving the liver suspension mixed with Rift Valley fever immune serum remained well; one mouse receiving the normal serum mixture survived, and one receiving the immune serum mixture died. This test showed that the infective agent in the serum of Lamb 6 was Rift Valley fever virus.

Further proof of the transmission was the observation that Lamb 6 developed protective antibody against Rift Valley fever virus as a result of the experiment (Table III). The incubation periods in this instance were: extrinsic, 20 days; intrinsic, 3 days.

Lots of E. chrysogaster *with which no transmission was effected.*—Thirteen *E. chrysogaster* group mosquitoes, Lot 3, received infective feeds from mice. Two of them (Table I) probed a normal mouse on the 10th day but got no blood; another bit a normal mouse on the 24th day, and one bit a lamb on the 13th day. No transmission occurred. Ten of the mosquitoes were tested individually for virus 13 to 25 days after their infective feeds. Six of these were positive, 4 on the 25th day out of 6 tested that day. In the light of other findings, the only opportunity for transmission with this lot was in the case of the mouse bitten on the 24th day, and we cannot be certain that the mosquito which bit that mouse contained virus.

Three other lots of *E. chrysogaster* group were included in the experiments. All the mosquitoes in 2 of the lots were dead by the 15th day, none having bitten normal animals after the 5th day; none of the third lot bit normal animals after the 18th day. No transmissions occurred. The 30 mosquitoes of the 3rd lot (Lot 12) received their infective feeds from Lamb 3, but after the peak of circulating virus in the animal was past, and when the titre of virus in its serum was 1 in 69, representing only 1152 effective (for mice) units of virus per ml. of serum. Each of the 30 insects in this lot was tested individually for virus. Only 6 of the 30 tests were positive, in sharp contrast with the results obtained with Lot 11, which fed on the same lamb at a time when the serum of the animal contained 567,800 LD_{50} of virus per ml. of serum, and in which 33 of 42 mosquitoes tested were found to have retained the virus. Thus the causes of failures in transmission were probably the following: (1) failure of the insects to bite normal animals after an appropriate extrinsic incubation period, and (2) low levels of virus in the serum of the animals which were used to infect the mosquitoes.

Table 4 shows the consolidated data on all the lots of the *E. chrysogaster* group, and of other species of mosquitoes tested, and includes the results of tests for virus by inoculation as well as results of the successful and unsuccessful

TABLE IV.—*Summary of Tests for Virus by Inoculation, and of Attempts to Transmit Rift Valley Fever Virus with 4 Species of Mosquitoes.*

Species of mosquitoes.		*Aëdes aegypti.*	*Eretmapodites chrysogaster* group.	*Taeniorhynchus fuscopennatus.*	*Taeniorhynchus uniformis.*
Number of lots		4	7	12	3
Number of mosquitoes		58	177	644	79
Tests for virus by inoculation into mice	Number*	49(49)	121(122)	263(348)	15(15)
	Positive	6	78†	40	6
Latest test for virus, day		37	37	38	14
Latest positive test for virus, day		26	31	29	13
Survival after infective feed, days.	Minimum	1	1	1	1
	Maximum	36	36	37	13
	Mean	15·2‡	14·4§	7·4	5·2
Mosquitoes alive 20th day	Number	25	52	34	0
	Per cent	43·1	29·4	5·3	0
Last biting, day		34	31	34	0
Number of mosquitoes biting after 19th day	All lots	63	18	77	0
	Better lots	3	18	34	0
Successful transmissions		0	4	0	0
Incubation period, days	Extrinsic	..	19,20	..	..
	Intrinsic	..	3–5	..	..
Lots failing to show virus‖		2/32	0/–	3/75	2 lots not tested.

* Figures in parentheses are numbers of mosquitoes included in the tests.

† 24 of 30 in one lot were negative. Excluding these, the number retaining virus was 72 out of 91. The one poor lot was infected on a lamb after the peak of circulating virus was passed.

‡ 7 mosquitoes were sacrificed on the 29th day, thus reducing the mean.

§ 19 mosquitoes were sacrificed 20 and 24 days after the infective feeds, thus reducing the mean.

‖ Numerator shows the number of lots, the denominator the number of mosquitoes tested in these lots.

attempts to transmit by bite. It will be noted that the *E. chrysogaster* group mosquitoes retain the virus much better than other species, and that they alone were successful in transmitting it.

Experiments with other species.

Transmission experiments with 4 lots of *Aëdes aegypti*, 12 lots of *Taeniorhynchus fuscopennatus*, and 3 lots of *Taeniorhynchus uniformis* which had taken blood from infected mice, were unsuccessful. Three of the 4 lots of *A. aegypti* tested may be dismissed from consideration, as they evidently got little or no virus with their infective feeds. Five of the 8 mosquitoes tested in the remaining lot (Lot 5) contained virus, but normal mice bitten by insects of this lot on the 5th, 7th and 12th days after their infective feeds, and a normal lamb bitten on the 10th, 14th, 18th, 21st and 24th days (Table I), did not become infected. The mosquitoes usually fed avidly on either the mouse or the lamb offered. Their failure to transmit to mice could have been due to the fact that insufficient time had elapsed between the infecting feed and the latest attempt to transmit. This was probably not the case with the lamb, however, as this animal was bitten as late as the 24th day after the mosquitoes fed, and the tests for virus in the insects of this lot showed that the lamb was bitten on every occasion by 1 or more mosquitoes which were proved to contain virus. From this evidence we conclude that *Aëdes aegypti*, if it can transmit Rift Valley fever at all, is a very poor vector.

Only 2 of 12 lots of *T. fuscopennatus* which fed on infected mice could be regarded as suitable for transmission experiments. The other 10 lots either succumbed *en masse* before transmission could have been expected to occur, or

the tests by inoculation failed to show more than a very small percentage of the mosquitoes to have retained the virus. Even the remaining two lots could not be considered good, as only 16 of 71 tests for virus in Lot 15 and 10 of 98 in Lot 17 were positive. Although mosquitoes of these 2 lots bit normal mice on 6 occasions up to the 13th day after their infective feed, they failed to transmit the virus, possibly because of an inadequate period of incubation. The 6 bitings of a normal lamb by Lot 15 (Table I) were all by the same mosquito, which contained no virus when it was found dead on the 38th day. A test for virus in a mosquito of Lot 17 found dead on the 29th day was positive, so that there was at least one infected insect in the lot each time the mosquitoes bit the lamb up to and including the 27th day. Whether or not the lamb was ever bitten by a mosquito containing virus cannot be stated. The mosquitoes that bit the lamb on the 29th and 31st days gave negative results in tests for virus.

No tests for the presence of virus were made in 2 of the 3 lots of *T. uniformis* which had fed on infected mice, and no mosquitoes of these 2 lots bit normal animals during the maximum period of 12 days that any of them lived. None of the mosquitoes of the other lot were exposed to a lamb, and none could be induced to take blood again from a mouse. All were dead by the 14th day. Fifteen mosquitoes of this lot were tested individually for virus as they died, and it was found to be present in 6 of these (Table IV). The longest period of demonstrated retention of the virus was 13 days.

Susceptibility tests on animals used in unsuccessful transmission.

Experiments.—The lambs employed in unsuccessful attempts at transmission were tested for susceptibility at the end of the experiments, either by inoculation with fully virulent pantropic virus or by serum protection tests. None was immune. All the mice bitten by mosquitoes, other than the 2 previously mentioned as having been infected, were given intraperitoneal test inoculations of virus at the end of the experiment. All were shown to be fully susceptible. It is therefore known that the 4 transmissions which we have described were the only ones that occurred.

Multiplication of virus in mosquitoes.

No tests were done with the express purpose of determining whether Rift Valley fever virus multiplies in any of the species of mosquitoes included in our experiments. Nevertheless, the tabulation of the results of inoculation tests with mosquitoes of the most satisfactory lots of each species, by intervals between infective feed and test for virus, gave information on this point which may be significant (Table V). It was seen that a high percentage of mosquitoes of the *E. chrysogaster* group harboured virus throughout the experiments. This does not prove that the virus multiplies in the group; but if it does not multiply, it must be exceedingly well maintained. That it actually does multiply in these mosquitoes is suggested by their ability to transmit the virus, but only after an extrinsic incubation period of 19 or 20 days.

Among the mosquitoes of the 2 species of *Taeniorhynchus*, on the other hand, the percentage containing virus was highest in the first 10 days after the infective feeds and then declined. This seems to indicate that, although a small proportion of *Taeniorhynchus* may maintain the virus for several weeks, in most mosquitoes of the species tested the virus slowly dies off in the insect without multiplying.

TABLE V.—*Summary of Results of Tests for Virus in Better Lots of Each Species of Mosquitoes by Intervals of* 10 *Days after Infective Feeds.*

Interval between infective feed and test (days).	*A. aegypti*, Lot 5.		*E. chrysogaster* group, Lots 1, 3, 7 and 11.		*T. fuscopennatus*, Lots 15 and 17.		*T. uniformis*, Lot 19.	
	Tested.	Positive.	Tested.	Positive.	Tested.	Positive.	Tested.	Positive.
1–10	2	1	22	16	117	22–1†	11	5
11–20	1	1	36	31	39	2	4	1
21–30	5	3	29	21–1*	11	1	0	..
Over 30	0	..	1	1	3	0	0	..

* 21 individual mosquitoes and 1 pool of 2 were positive, so that 22 or 23 contained virus.
† 22 individual mosquitoes and 1 pool of 2 were positive, so that 23 or 24 contained virus.

The *Taeniorhynchus* mosquitoes used in these experiments took their initial (infective) feeds well. Only mosquitoes which showed no visible blood were exposed to infected animals. The immobilized mouse could then be left in the Barraud cage unattended, and the mosquitoes which had taken blood could be easily identified when inspected a few hours later. A high percentage of those exposed to infected animals obtained blood within a few hours. However, it was only with difficulty and the exercise of considerable patience that many of them could be induced to take blood again for a transmitting feed. Moreover, the mosquitoes of this genus did not thrive well in captivity (Table IV), and it was difficult to keep them alive long enough to carry out the necessary procedures. Whether these mosquitoes are short lived in nature, or are ill suited to the environment of the laboratory, is unknown, but it seems possible that their aversion to repeated blood meals is a characteristic of the genus.

The *A. aegypti* tested were too few in number to permit conclusions with regard to multiplication of the virus in this species.

Entomological note on the E. chrysogaster *group in the Kitinda area.*

The *Eretmapodites chrysogaster* Graham group mosquitoes used in the transmission experiments were wild females caught in lake-side forest at Kitinda, about 2 miles west of Entebbe. The *E. chrysogaster* group as defined by Edwards (1941) contains 5 species; no wholly reliable characters are known by which the females of the group may be separated one from another. For the determination of the actual species used it was, therefore, essential to examine males derived from the same locality. Of 40 wild males collected there between December 11, 1947 and January 17, 1948, 37 were *E. chrysogaster* Graham and 3 were *E. intermedius* Edwards. It was considered possible, however, that wild females caught biting in the same locality might be of different species, so females were collected for egg-laying in order that males might be bred from them for examination. They were repeatedly offered blood meals in the laboratory. Those which fed were kept at about 24° C. for 48 hours in Barraud cages covered with damp cloths, and were allowed access to 5 per cent glucose solution. They were then isolated individually in glass tubes (15 × 85 mm.) lined with moist filter-paper and plugged with cotton. The tubes were stored at 30° C. until eggs were laid or the females died. Batches of eggs laid by individual females were hatched separately; the larvae were mainly fed with appropriate instar larvae of *Aëdes* (*S.*) *aegypti* from a stock culture. *Eretmapodites* males hatching from these cultures were then examined to determine the identity of the original female which laid the eggs. In all, 608 females were collected between November

10, 1947 and January 19, 1948, and of these, 139 laid eggs. However, a considerable proportion of these egg batches failed to hatch, and only 48 were successfully reared through to yield adult males for examination; i.e., of the original 608 females, only 48, or 7·9 per cent, were identified by the male characters of their progeny. However, all these 48 were found to be *E. chrysogaster*. Although the proportion definitely identified is small, the fact that all were *E. chrysogaster*, taken together with the great preponderance of this species among the wild males, indicates that the *Eretmapodites chrysogaster* group population at Kitinda is composed almost entirely of *Eretmapodites chrysogaster* Graham itself, with only a small component of *Eretmapodites intermedius* Edwards.

We are indebted to Mrs. E. C. C. van Someren for the determination of all but 18 of the terminalia examined; the remaining 18 have been identified by comparison with the material determined by her, and were all *E. chrysogaster*.

DISCUSSION.

The circumstances of the outbreaks of Rift Valley fever affecting humans and domestic animals on farms in Kenya (Daubney and Hudson, 1931, 1933), and those of the outbreak in the Semliki Forest in Bwamba County, Uganda (Smithburn, Haddow and Gillett, 1948), were sufficiently different to indicate that the vector insects probably were not the same. The country concerned in Kenya is open table land at an altitude of about 5000 feet, whereas the affected locality in Uganda is uninhabited virgin forest at an altitude of about 2500 feet. Daubney and Hudson (1933) found that the protection of susceptible animals from mosquito bites during the hours of darkness alone sufficed for almost complete prevention of the infection, and this was strong evidence in favour of a night-biting vector. On the other hand, all the mosquitoes from which virus was isolated in the Semliki Forest outbreak were taken on human baits during the hours of daylight. Furthermore, the mosquitoes here incriminated as the probable vectors in that outbreak, the *Eretmapodites chrysogaster* group, are, in Uganda, day-biting insects which are not commonly taken in night catches. Finally, it seems most improbable that this sylvan mosquito could propagate in the open country where the Kenya epidemics occurred.

The aforementioned facts, plus the apparent demonstration of virus in wild-caught *Taeniorhynchus fuscopennatus* in Kenya (Daubney and Hudson, 1933), caused us to become interested in the genus *Taeniorhynchus*, most, if not all, of the members of which are night-biting mosquitoes. In the original paper of Daubney and Hudson (1931) this mosquito was called *Taeniorhynchus brevipalpis*, while in a subsequent communication (1933) it was designated *Mansonia fuscopennata*. Throughout the present paper we have used the terminology of Edwards (1941). The 2 species studied here, *T. fuscopennatus* and *T. uniformis*, have not yet been ruled out as possible vectors owing to the difficulty encountered in keeping them alive and in inducing them to take blood at any time after the first (infecting) feed. Nevertheless, the tests for virus in mosquitoes of these 2 species showed a low percentage infected, and an apparent decline in the percentage containing virus with increasing passage of time after the infecting feed. Neither *T. fuscopennatus* nor *T. uniformis* has, to our knowledge, ever been incriminated as the vector of any disease. However, another member of

this genus, *Taeniorhynchus* (*Mansonioides*) *africanus* Theo., has been shown to be capable of transmitting yellow fever (Philip, 1930), and this or some other as yet untested species of the genus may be capable of transmitting Rift Valley fever.

The occurrence of Rift Valley fever in the Semliki Forest, doubtless involving as host(s) some species of wild animal, the isolation of the causative virus from sylvan mosquitoes, and the subsequent experimental transmission of the disease by insects of one of the species from which the virus was isolated, not only places Rift Valley fever among the diseases which can be transmitted by insects, but associates it as well with the increasing group of diseases having cycles of infection which do not include man or domestic animals.

While the experiments here reported do not incriminate a single species of mosquito as a vector of Rift Valley fever virus, they do show that at least one of the *E. chrysogaster* group can transmit it. The mosquitoes of this group are so closely related and so alike in their habits that it seems possible that the whole group may possess vector potentialities.

SUMMARY.

Rift Valley fever virus was transmitted experimentally from mouse to lamb, from mouse to mouse, from lamb to lamb, and from lamb to mouse by the bites of mosquitoes of the *Eretmapodites chrysogaster* group. The success of these experiments indicates that these mosquitoes, which were included in the *Eretmapodites* spp. from which the virus had previously been isolated, were probably responsible for the transmission of the virus in the Semliki Forest in 1944. The period of incubation in the *E. chrysogaster* group was 19 or 20 days at 30° C.

Attempts to transmit the virus with *Aëdes aegypti* and with 2 species of the genus *Taeniorhynchus* were unsuccessful. It seems probable that the former cannot transmit this virus, but the experiments with *Taeniorhynchus* were inconclusive owing to technical difficulties. The virus may survive for a number of days in these mosquitoes, and it is possible that one or another species of this genus can serve as a vector.

The results seem to place Rift Valley fever definitely in the group of insect-borne virus diseases.

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FACTORS INVOLVED IN INFLUENZA HAEMAGGLUTINATION REACTIONS.

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THE original object of our investigation was the study of any fluctuations of serum antibody level which might occur in individuals during inter-epidemic periods. The scale of the intended survey and the necessity for detecting relatively small antibody variations determined the choice of the haemagglutination test for our purpose. It was recognized that this test is complicated by variables pertaining to influenza viruses, chicken red blood cells and serum constituents other than specific antibodies, but the claims of previous workers and the precision of measurement attainable by means of a photo-electric densitometer encouraged the hope that variables might be sufficiently controllable to allow reasonably accurate antibody assays. The end in view has not yet been achieved, but unsuspected factors have been brought to light which may have an important bearing in work based upon the haemagglutination reaction.

MATERIALS AND METHODS.

Haemagglutination tests.

The technique employed was essentially that of Hirst and Pickels (1942), in which the degree of fowl red cell agglutination occurring in test mixtures is measured by the amount of light transmitted through the mixtures from a light source of constant intensity on to a photo-electric cell. Instead of a microammeter we used a sensitive mirror-galvanometer with scale readings from 0 to 100, and the light intensity was standardized at the beginning of each test so that 50 per cent of the test mixture concentration gave a galvanometer reading of exactly 50. Thus, the reading 50 invariably corresponds to 50 per cent haemagglutination, which has been adopted as the end-point in all titrations. Provided that galvanometer readings above and below 50 are obtained, determination of the virus or serum dilution corresponding to this end-point is a matter of simple calculation. Titres are expressed throughout as the reciprocals of such dilutions.

For all serum titrations a standard dose of 4 agglutinating doses (4 AD) of virus was employed. To obtain this dose all virus preparations to be used in a test were titrated immediately prior to the test. Such preliminary titration is absolutely essential because of the variability of red blood cells of different fowls (Stuart-Harris 1943; Stuart-Harris and Miller, 1947) and the rapid decline of sensitivity of red blood cells during storage (Miller and Stanley, 1944), both of which phenomena we have amply confirmed. Moreover, the responses to heat treatment of different batches of a virus, or even of the fluids from different eggs of the same batch, may be far from uniform.

Viruses.

Influenza viruses A (PR8 strain), B (Lee strain) and Swine (Shope strain 15) were maintained by serial egg passage using the allantoic route of inoculation. At the beginning of the investigation batches of each of the viruses were laid down in cold storage (— 20° C.) to serve as reference standards; in each case pooled allantoic fluid virus was distributed in convenient quantities in sealed ampoules. These stored preparations are referred to as "stock" viruses. "Fresh" virus preparations were also used; they consisted of allantoic fluid harvested usually within 24 hours of the test, but sometimes a few days previously, and kept at approximately 4° C. Gross particles were removed from virus fluids by centrifugation.

Sera.

Immune horse sera to each of the viruses were distributed in sealed ampoules and kept at — 20° C. as "stock" sera. The A and B sera were reconstituted from freeze dried preparations kindly supplied by Dr. C. H. Andrewes; the Shope serum was from a horse immunized in 1934 and stored since that time at approximately 4° C.

Human sera were obtained from volunteers. If not required fresh they were inactivated in sealed ampoules at 56° C. for 30 min. and stored at — 20° C. without addition of any preservative.

Relevant details in respect of sera of other species are given in the text and protocols.

Cholera enzyme.

A potent enzyme preparation was obtained from agar cultures of *V. cholerae* by the method of Burnet and Stone (1947), and was purified by adsorption on, and elution from, human red blood corpuscles. Crude filtrates were quite unsuitable for our purpose because of their high content of proteinase, which caused destruction of specific serum antibodies. The red blood cell eluate, however, showed no proteinase activity by the azocoll test (Oakley, Warrack and van Heyningen, 1946), although about two-thirds of the original receptor destroying enzyme (R.D.E.) activity was retained. The eluate was distributed in ampoules in 0·5 ml. quantities and freeze dried.

EXPERIMENTAL.

1. *Virus Degradation as a Factor in Serum Antibody Titration.*

Instability of virus at — 20° C.

Both the infectivity and the haemagglutinating activity of influenza viruses decline rapidly at room temperatures and more slowly at 4° C., but our reference stocks had been laid down on the assumption that virus kept in the frozen state at — 20° C. would remain stable. Certain unexpected results obtained with the stock viruses led to a number of comparative titrations which are summarized in Table I. All tests at a stated period of storage were done in parallel with the same red cell suspension, so that titres in each vertical column of the table are comparable, whereas differences along horizontal rows are, of course, without

any significance. It is evident that freezing and storage at — 20° C. result in appreciable degradation of the PR8 virus, some of which occurs during the actual freezing process. Miller and Stanley (1944) found that virus remained stable for several months at 4° C., more stable in fact than when kept at — 70° C. We have been unable to confirm this, for in our experience steady degradation has always occurred at 4° C., and some experiments recorded later provide evidence that the change is not only a question of reduction of haemagglutination activity but also of qualitative behaviour. It is probable that such discrepancies are due both to strain differences and to differences in physical states of the virus-containing fluids.

TABLE I.—*Instability of Influenza Virus PR8 at — 20° C.*

Temperature ° C. of-		Haemagglutination titres after various periods of storage.				
Freezing.	Storage.	30 min.	15 days.	32 days.	32 days.*	9 months.
—76	—76	561	320	..	..	364
—76	—20	..	..	160	281	..
—20	—20	444	160	134	101	269
..	4	501	403	160	222	180
..	R.T.	493	..	..	..	..

* Glycerinated virus.

Heat inactivation of virus.

Francis (1947) showed that many sera contain a substance which inhibits haemagglutination by killed influenza B viruses but not when living virus is used. Thus, the apparent antibody titre of a serum may be much higher against killed virus than against living virus—an effect which we refer to as the Francis phenomenon or Francis effect. It is now generally accepted that the phenomenon is due to a heat-labile virus enzyme which destroys red cell receptors and which also rapidly destroys the Francis inhibitor. In early work with the PR8 influenza virus we encountered the same phenomenon. Whilst it is probable that the Francis inhibitor substance is effective against A viruses as well as against B viruses, there is, as yet, no proof of this and, as will be shown later, sera differ qualitatively in respect of their inhibitory properties, indicating the existence of several inhibitory substances of the Francis type. All the substances in sera effective against killed PR8 virus are therefore referred to as "alpha inhibitor," with full recognition of the likelihood that future work will lead to their separation and characterization.

Because of the presence of such non-specific inhibitory substances in many sera, an analysis of the factors concerned in antibody titration demands the use of "inactivated" viruses capable of revealing their presence. Inactivation has usually been by heating at 56° C. for 30 min. but occasionally additional short treatment at 60° C. has been used. Different virus strains have different degrees of susceptibility to heat (Salk, 1946), and the necessary destruction of virus enzyme may sometimes be achieved without any reduction of haemagglutinin activity (Briody, 1948). In the present study, however, the viruses employed have invariably suffered a large reduction of haemagglutination titre on heating to 56° C. for 30 min.; indeed the Shope virus has always been completely inactivated by such treatment.

PR8 virus has shown a striking variability in behaviour from one experiment to another, so that the degree of haemagglutinin reduction was never predictable ; it appeared to depend not only upon the initial titre of the untreated virus, but also upon the age of the virus preparation since harvesting and possibly the sensitivity of the red blood cell suspension employed. Thus, stock virus usually suffered a 50 to 75 per cent reduction, whilst freshly harvested allantoic fluids often suffered less than 25 per cent. These points are illustrated in Table II.

TABLE II.—*The Effect of Heat Treatment, 56° C. for 30 min., on Virus Haemagglutination Activity.*

Virus.	Haem. titres.		
	Untreated.	Heated.	% Decrease.
PR8	88	34	61
,,	131	38	71
,,	160	40	75
,,	400	148	63
,,	184	56	70
,,	160	80	50
,,	92	63	32
,,	90	81	10
,,	84	54	36
,,	435	218	50
,,	226	135	40
,,	123	55	55
Lee B	125	53	58
,,	188	124	34
,,	160	110	31
,,	180	106	41
,,	142	88	38
,,	80	70	12
Shope	114	0	100
,,	217	0	100
,,	213	0	100

More important than this variability of haemagglutinin reduction is the possibility that the heat treatment may occasionally fail to inactivate completely the virus enzyme which destroys alpha inhibitor, so that the presence of the inhibitor in a serum is masked. The temperature and time of inactivation employed, namely 56° C. for 30 min., have been generally accepted as entirely sufficient for the purpose and Briody (1948) found that a still lower temperature sufficed, but doubt arose when a human serum showed no Francis effect on one day, but did so to a high degree when retested a few days later with older virus from an earlier egg passage. To settle the matter the serum was then titrated against the two virus fluids at the same time and with the same reagents. The previous results were confirmed, although the " fresh " virus was at the time of test already 4 days old. The experiments are summarized in Table III.

The titration of the standard PR8 serum against living and heated PR8 virus consistently failed to show the Francis phenomenon. This was not sur-

TABLE III.—*The Effect of Different Batches of Virus upon the Results of Serum Titrations.*

Expt. No.	Date.	Virus batch.	Titres of virus.		Titres of human serum "MS" against 4AD's of-	
			Untreated.	Heated.	Untreated virus.	Heated virus.
1	18/vi/48	PR8/AL 44	726	415	65	69
2	21/vi/48	PR8/AL 42	190	98	60	435
3	22/vi/48	PR8/AL 44	433	380	56	77
		PR8/AL 42	186	127	70	>1280

prising, for some sera are devoid of the non-specific inhibitor substance. Repeated tests, however, with these stock reagents brought to light a phenomenon exactly the reverse of that of Francis, namely, that some sera exhibit a lower titre of haemagglutination inhibition against heated virus than against fresh living virus, especially if the latter is of high titre. With PR8 standard serum the differences were never large, but the consistency of the results of very many experiments indicated significance.

A similar difference was obtained in each case with a number of immune ferret sera. With one of them (F 324, Table IV) a four-fold difference of antibody content was apparent according to whether untreated or heated virus was employed for the titration. On the other hand, a fresh normal ferret serum, normal rabbit sera, and all fresh human sera tested have given pronounced Francis effects.

TABLE IV.—*Effect of Virus Degradation upon Serum Antibody Titres.*

Serum.	Serum titres with 4 AD's of PR8 virus.		Per cent decrease.
	Untreated.	Heat killed.	
PR8 stock	370	253	32
,, ,,	403	238	41
,, ,,	372	236	36
,, ,,	280	235	16
Immune Ferret 255	1920	1285	33
,, ,, 319	2358	1600	32
,, ,, 320	2751	1600	42
,, ,, 321	>3200	2436	>24
,, ,, 322	2540	1942	24
,, ,, 323	>3200	2580	>19
,, ,, 324	2540	580	77

This reduction of apparent antibody titre may be due partly to the increased bulk of virus protein contained in the 4 agglutinating doses of virus used, for the reduced haemagglutinin titre of heated virus necessitates the employment of larger quantities of virus fluid than in the case of untreated virus of high titre. If virus bulk is indeed a factor of importance the proportions of living and dead virus in any preparation used for a serum antibody titration will affect the result, no matter how carefully the preliminary titration of the virus for the estimation

of 4 agglutinating doses has been carried out. This is supported by the results of serum titrations in parallel against mixtures of varying proportions of untreated and heated virus. As the proportion of heated virus is increased the serum titre steadily declines until not enough living virus is incorporated in the mixture to supply a critical dose of inhibitor-destroying enzyme. At this point sera containing alpha inhibitor show a sudden rise of titre, while those devoid of inhibitor show still further decline.

The behaviour of the ferret sera, shown in Table IV, however, indicates that virus bulk cannot be the only factor concerned, because of two sera tested at the same time and with identical titres against living virus, one of them (F 324) shows over 75 per cent reduction, the other (F 322) less than 25 per cent. Thus, the difficulty cannot be resolved by estimating serum antibodies in terms of the ratio of activity between the test serum and a standard serum. The ratios of the ferret sera in question would obviously depend upon whether untreated or heated virus were employed, and possibly, in the former case, upon the proportions of living and dead virus present. Which of the ratios would be more nearly indicative of the true *in vivo* virus neutralizing power it is impossible to say without further information from extensive correlation studies.

2. *Haemagglutinin-inhibitory Substances of Normal Sera.*

Species differences.

The investigations reported above raised the suspicion that inhibitory substances of the Francis type in sera might affect results of antibody titrations even if fully active living virus were used; also that such substances might play some part in host resistance against natural infections with viruses of the influenza group. Investigations of "normal" human sera are complicated by the fact that, in addition to non-specific inhibitory substances, most of them contain specific virus-neutralizing antibodies which are believed to reflect previous influenza infections, either overt or sub-clinical. The sera of several other animal species were therefore examined. All were tested against both A and B influenza viruses, living and heat killed; in addition some were tested against swine influenza virus, but in this case only living virus could be used because of the complete destruction of its haemagglutinins by heating at 56° C. for 30 minutes (Table II). Representative tests are recorded in Table V.

TABLE V.—*Inhibition of Virus Haemagglutination by Normal Sera of Various Species.*

Serum.	Serum titres with 4 AD's of viruses.				
	PR8 living.	PR8 killed.	Lee B living.	Lee B killed.	Swine living.
Guinea-pig	147	267	241	926	<40
Rabbit	32	236	61	370	<20
Mouse	76	46	83	83	<20
Rat	28	40	..	..	..
Ferret	50	92	30	1085	..
Horse	0	380	0	320	..
Sheep	64	287	72	1024	>32
Chicken	60	202	20	587	21
Plaice	20	640	25	>1280	<20

Only single samples of sheep, chicken and plaice sera were available, but with the other species listed several individual sera were tested, all of which behaved like the representative recorded in the table. It is clear that most species possess inhibitory serum constituents of the Francis type, effective against both A and B influenza viruses. Differences in the magnitudes of the Francis effects obtained in different experiments are entirely without significance, for this largely depends upon the particular batch of virus used; indeed, with freshly harvested virus of high titre, the effect may be abolished altogether (*cf.* Table III). The absence of the phenomenon in the cases of mouse and rat, however, is almost certainly due to a true lack of this particular type of inhibitory substance; sera of these species never show the effect, even when tested against virus batches particularly suitable for the purpose. The obvious inference is that other inhibitory substances of different type exist. This is also indicated by the fact that the sera of most species possess appreciable inhibitory power against living viruses of both types A and B; in curious contrast is the lack of similar activity against Swine influenza virus.

Amongst the species so far investigated the guinea-pig appears to occupy a unique position. Every guinea-pig serum tested has given remarkably high titres against living viruses, titres indeed of the same order as those given by adult human sera which contain specific antibodies. This led us to test three guinea-pig sera for virus neutralizing antibodies in mouse neutralization tests in parallel with sera of other species. The usual method of titrating serial dilutions of serum against a constant amount of virus containing many lethal doses cannot be expected to reveal small amounts of antibody; we therefore tested various dilutions of virus filtrate mixed with equal quantities of serum. The experiments are summarized in Table VI. All three guinea-pig sera showed evidence of considerable virus neutralizing power; one of them neutralized over a thousand minimal infective doses of virus. In Experiment 3 a sample of the guinea-pig serum inactivated by heating at 56° C. for 30 min. was also tested. The heat treatment caused almost complete abolition of neutralizing power, a result directly contrary to what was expected in view of the effect of inactivation upon haemagglutination inhibition described below. One obvious possibility is that complement may be required for the *in vivo* neutralization, although, so far as is known, complement does not participate in the neutralization of viruses by their specific antibodies. This aspect of the work is under further investigation, and will be more fully reported in a subsequent paper.

Effect of heat inactivation of sera.

It is customary to inactivate by heat treatment all sera intended for antibody titration. The original purpose of this was, of course, destruction of complement which would obscure results of complement-fixation tests; in addition it provides some safeguard against bacterial contamination. The possible effects of such heat treatment upon the anti-haemagglutination titres of immune sera appear to have been overlooked, whilst in the case of normal sera published reports are directly contradictory (McCrea, 1946; Hirst, 1948). This is not surprising in view of the complexity of the factors involved. The experiments summarized in Table VII confirm the existence of quantitative differences in respect of alpha inhibitor between various animal species, but they also reveal qualitative

TABLE VI.—*Mouse Neutralization Test—Neutralization of Influenza Virus by Guinea-pig Sera.*

Expt. No.	Serum 1/2 dil.	Dilutions of PR8 virus filtrate.																					
		1/20			1/200			1/2 × 10^3			1/2 × 10^4			1/2 × 10^5			1/2 × 10^6					
1	Guinea-pig A	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	..	..	..	..	..	..	..
	Horse	D.	D.	+++	D.	D.	+++	+++	+++	+	+	±	0	+	+	0	..	..	..	..	..	..	..	..
2	Guinea-pig B	++	+	+	+	+	0	0	0	0	0	0	0	..	..	..	..	..	..	..	..	..	..
	Horse	..	..	..	..	..	..	D.	+++	+++	++	++	++	+	0	0	0	0	0	0	0	0	0
	Rat	..	..	..	..	..	..	+++	+++	+++	++	++	++	+	0	0	0	0	0	0	0	0	0
	Rabbit	..	..	..	..	..	..	+++	++	++	++	+	0	0	0	0	0	0	0	0	0	0	0
3	Guinea-pig C	+++	+++	+++	+	±	0	±	0	0	..	..	..	..	..	..	..	..	..	..	..	..	..
	Guinea-pig C Inactivated	D.	D.	D.	D.	D.	D.	+++	+++	++	++	+	+	..	..	..	..	..	..	..	..	..	..
	Horse	..	..	..	..	..	..	D.	+++	+++	+++	+++	++	++	+	+	..	..	..	..	..	..	..

D. = Mouse died with almost complete lung consolidation.
+++, ++, +, ± indicate diminishing degrees of lung involvement.

differences. In tests against living virus the effect of heat inactivation of the sera of all species is to reduce their inhibitory power. Tests against killed virus, however, separate the species into distinct categories. Some, like mouse, yield sera which always suffer reduction of titre on heat inactivation; others, like rabbit, have sera which may show either reduction or slight increase of doubtful significance, whilst guinea-pig sera invariably show a large increase which may amount to as much as a tenfold rise. This peculiar property of guinea-pig sera cannot be due merely to an exceptionally high content of alpha inhibitor because rabbit sera containing just as much may show the contrary effect.

TABLE VII.—*Heat Inactivation, 56° C. for 30 min., of Normal Sera of Different Species.*

Serum.	4 AD's living PR8 virus with—		4 AD's killed PR8 virus with—	
	Fresh serum.	Heated serum.	Fresh serum.	Heated serum.
Guinea-pig 1	178	213	470	5120
,, 2	172	121	1280	3482
,, 3	286	181	806	5120
Mouse 1	59	0	42	0
,, 2	76	0	46	0
,, 3	>128	41	111	18
Rabbit 1	32	20	236	177
,, 2	67	32	415	237
,, 3	54	34	1350	1670
,, 4	64	45	1561	1881
Ferret	50	48	92	119

The effect of heat inactivation upon adult human sera is quite unpredictable. As with other species, it always results in reduction of titre against living virus, but against killed virus there may be reduction, increase or no significant change. Samples obtained from an individual donor at different times usually all show the same type of effect, but this is not invariably the case. There is also some evidence that the demonstration of increased activity on heat treatment is partly dependent upon the degree of susceptibility to virus agglutination of the chick cells employed in the test. A full explanation of these peculiarities of human sera has not been found, but it seems probable that they depend upon the relative concentrations of specific antibodies and non-specific inhibitor substances. Whilst in the absence of further antigenic stimuli the concentration of specific influenza antibodies in a person's serum remains fairly constant over a long period, the concentration of other haemagglutination inhibitor substances may undergo considerable fluctuations.

Fluctuations of serum inhibitor substances in human volunteers.

During the period October, 1947, to May, 1948, 21 volunteers, chiefly medical students, were bled at intervals of one to two months. From each sample the serum was separated, inactivated by heating at 56° C. for 30 min. in a sealed ampoule, and stored at — 20° C. until required for test. All the samples from each individual were tested in parallel at one and the same time against both

living and heat-killed PR8 virus. Most of the volunteers gave four samples, but some gave only two or three. The results of all the tests are tabulated in Table VIII. A surprising degree of fluctuation is shown by most of the sets of sera. As was to be expected, the serum changes are best revealed by tests against killed virus, for they probably consist chiefly of fluctuations in concentration of alpha inhibitor. Titre differences up to 30 per cent may be due to the experimental error of the test and are without any significance, but no less than 16 of the 21 volunteers yielded sets of sera giving over 50 per cent differences, and 11 sets show over 100 per cent differences. Still more surprising is the fact that 9 sets of sera show over 50 per cent differences in the tests against living virus. None of the volunteers suffered any illness resembling influenza during the sampling period, so that there appears to be no escape from the conclusion either that alpha inhibitor may affect the results of serum antibody titrations even when living virus is used, or that other serum constituents, more akin to the specific antibodies and not destroyed by the virus enzyme, may increase and decrease from time to time quite independently of any specific antigenic stimulation. Possibly both explanations are true, but the hypothesis that serum titres in the standard type of test with living virus may be partly

TABLE VIII.—*Serum Titre Fluctuations in Human Volunteers.*

Serum donor.	Living PR8 virus. Serum samples.						Killed PR8 virus. Serum samples.					
	1.	2.	3.	4.	H/L	% Inc.	1.	2.	3.	4.	H/L	% Inc.
S. A—	139	138	144	133	1·1	8	181	187	170	148	1·3	26
E. H—	213	181	177	171	1·2	24	192	177	235	168	1·4	39
C. E—	>640	448	380	497	1·7	68	1228	392	240	413	5·1	411
D. M—	93	52	61	55	1·8	78	320	95	127	77	4·2	315
M. W—	40	<40	<40	..	1·0	..	452	190	242	..	2·4	137
J. M—	110	82	127	85	1·5	54	359	307	250	247	1·4	40
E. L—	320	121	242	132	2·6	164	2560	508	1280	761	5·0	403
S. B—	94	106	68	94	1·6	55	1689	1689	537	1280	3·1	214
M. P—	128	95	45	75	2·8	184	4304	4362	830	1280	5·3	425
L. G—	50	52	51	..	1·0	4	1016	2152	735	..	2·9	192
P. T—	87	62	..	..	1·4	40	2228	1076	..	..	1·1	107
W. C—	58	58	..	..	1·0	..	254	137	..	..	1·9	85
D. F—	196	174	168	..	1·2	16	351	320	233	..	1·5	50
V. W—	72	85	101	..	1·4	40	195	320	452	..	2·3	131
F. K—	118	112	132	..	1·2	17	320	261	292	..	1·2	22
E. M—	160	101	88	89	1·8	81	640	735	462	538	1·6	59
E. B—	70	52	54	..	1·3	34	282	274	288	..	1·5	5
C. M—	103	82	62	109	1·8	75	970	508	493	707	2·0	98
J. H—	114	130	126	116	1·1	14	1280	951	844	485	2·6	163
M. M—	262	265	234	..	1·1	12	1902	1522	761	..	2·5	149
J. D—	74	103	116	116	1·6	56	345	640	545	640	1·9	85

H/L = Ratio of highest and lowest titres.
% Inc. = Percentage increase of highest over lowest titre.

determined by alpha inhibitor is strongly supported by the fact that, on the whole, the significant fluctuations shown in the tests with killed virus are reflected by corresponding smaller fluctuations against living virus.

The use of cholera enzyme in antibody titration.

Because of the instability of living influenza virus and the unpredictable behaviour of freshly harvested batches the use of killed virus for antibody assay

would be preferable, provided that all non-specific inhibitor substances could be removed from the sera to be tested. The possibility of achieving this by means of cholera enzyme has been explored. Since the completion of the work a recent publication by Mulder and van der Veen (1948) became available which reports the use of cholera enzyme for the same purpose but without giving details of the results of the treatment.

A purified enzyme preparation was used which showed no proteinase activity by the azocoll test. Equal quantities of the enzyme in calcium acetate buffer and serum were mixed, incubated at 37° C. for an hour and then heated at 56° C. for 30 min. to inactivate the enzyme. A control mixture of the serum and buffer solution was subjected to the same treatment in parallel. Preliminary tests showed that the buffer solution itself had no deleterious action on the inhibitory power of serum.

The results of experiments on three human and three normal guinea-pig sera are shown in Table IX. They indicate that the method may prove satis-

TABLE IX.—*Treatment of Sera with Cholera Enzyme.*

Serum.	Control or enzyme treated.	Serum titres with 4 AD's PR8 virus.	
		Living.	Heat killed.
Human W. S. . . .	C	42	109
	E	<32	<32
„ M. W. . . .	C	53	456
	E	40	44
„ Mar. . . .	C	64	254
	E	47	38
Normal guinea-pig 1 . .	C	640	9122
	E	293	3880
„ „ 2 . .	C	160	3225
	E	48	806
„ „ 3 . .	C	44	1762
	E	<40	320

C = Control sample. E = Enzyme treated sample.

factory provided that the potency of the enzyme used is adequate for removal of all alpha inhibitor contained in the serum. With the three human sera this was the case, for after the enzyme treatment titres against living and killed virus were not significantly different. These titres, however, were consistently lower than the tires of the corresponding control samples against living virus. The differences may possibly have been due to the concomitant destruction of some of the true specific antibodies by residual proteinase in the enzyme preparation which was not detected by the azocoll test. It is, however, much more probable that such reductions of titre represent the part played by alpha inhibitor in tests against living virus. The experiments with guinea-pig sera strongly support this view. Obviously the enzyme used was not potent enough to destroy all alpha inhibitor present in the guinea-pig sera, but in each case the effect of the enzyme treatment was to reduce titres against both killed and living virus by approximately the same proportionate amount.

DISCUSSION.

The importance of virus haemagglutination reactions is now well established, for by their agency considerable light has been shed upon some of the fundamental problems of infection. Their use, however, for the detection and measurement of serum antibodies is open to question. For such purposes the desirability of using standardized and stable virus is obvious, but it is doubtful whether this is possible with living virus preparations, for these have been shown to vary from time to time and from batch to batch, not only as regards their haemagglutination titres, but also qualitatively in a manner which affects the inhibitory potency of sera. The factor chiefly responsible for such instability may be the unpredictable and ever-changing proportions of living and dead virus. In any case heat-killed virus is much more likely to be both stable and standardizable, but it can be employed for antibody assay only in the complete absence of non-specific inhibitory substances of the Francis type. The possibility of removing such inhibitory substances from sera by treatment with cholera enzyme has been demonstrated by the few preliminary experiments reported, but before the method can be adopted rigorous proof is required that the treatment is without any deleterious effect upon specific antibodies, and that the titres of enzyme-treated sera obtained by haemagglutination show a close and constant correlation with titres obtained by mouse neutralization tests.

The alpha inhibitor and probably other serum constituents of like nature participate in influenza haemagglutination reactions even when living virus is used, and under some circumstances they may apparently affect the results of antibody titrations. This might be of little importance if all human sera contained approximately the same amount of non-specific inhibitors, or even if the serum of each individual remained constant. Such, however, is not the case; in this respect animal species vary, different members of each species vary, and the serum of an individual may fluctuate from time to time. The majority of the volunteers examined showed considerable fluctuations over a short period of a few months, in spite of the facts that the group was fairly uniform as regards age, social status and mode of life and that none suffered illness throughout the period of experiment. It is true that in this small series the maximum effect upon the serum inhibition titres of any individual was only a 2·8-fold increase when living virus was employed for the titrations, but it is probable that much greater serum changes may occur from time to time as a result of non-specific stimuli of various kinds. Indeed we have already obtained some evidence that febrile disturbances quite unconnected with virus infections may affect profoundly the concentration of alpha inhibitor in the patient's serum. Thus the usual interpretation of titre differences between acute and convalescent samples of serum, in both diagnostic and epidemiological work, is open to question.

Apart from their participation in haemagglutination reactions serum inhibitors of the Francis type may be of considerable importance. It is difficult to believe that serum constituents capable of blocking the virus receptors concerned in the initial stages of cell invasion are without any influence upon the susceptibility of the host to infection. In many virus diseases, including influenza, the behaviour of individuals and the epidemiological patterns of outbreaks are not always explicable on the basis of levels and distribution of specific antibodies in the population at risk. Non-specific factors which are completely

overshadowed in experimental laboratory work may well play a part in natural infections and may also be concerned in the determination of species susceptibility and resistance. It is in this connection that the *in vivo* neutralization of virus by normal guinea-pig sera may have special significance, though it is, as yet, quite uncertain whether their neutralizing power is connected with their exceptionally high concentration of alpha inhibitor. The two effects of heat inactivation, namely increase of haemagglutination inhibition titre against killed virus and loss of virus neutralization activity in mouse tests, suggest that serum constituents of different nature must be responsible for the two types of activity. Burnet and McCrea (1946) report similar high haemagglutination inhibition titres of normal ferret sera coupled with a virus neutralization activity which was peculiarly strain specific. In their case, however, the inhibitory substance was found by salt precipitation methods to be a part of the gamma globulin fraction of the serum proteins, and they assumed that the same component was responsible for the different types of activity. Obviously further investigations of the sera of different species by all available methods are indicated.

SUMMARY.

Influenza viruses suffer degradation by freezing and storage even at very low temperatures. Qualitative differences in different batches of virus may affect results of serum titrations.

Immune sera devoid of non-specific inhibitor substances of the Francis type (alpha inhibitor) give lower inhibition titres against heat-killed influenza A virus than against living virus. It is suggested that the bulk of virus protein contained in the standard haemagglutinin dose of virus employed may be partly responsible for the phenomenon.

The sera of all animal species tested were found to contain non-specific inhibitory substances. In addition to quantitative differences, species differ in respect of the qualitative behaviour of their serum inhibitors. Guinea-pig sera appear to be especially rich in such substances.

Of three normal guinea-pig sera tested in mouse neutralization tests all showed virus neutralizing potency.

Considerable fluctuations of the concentration of non-specific serum inhibitory substances occur from time to time in normal healthy human beings. Such changes may affect the results of influenza antibody titrations by the haemagglutination reaction even if living virus is employed.

The removal of alpha inhibitor from sera by means of cholera enzyme may facilitate the accurate assay of influenza antibodies in human sera.

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THE EFFECT OF HEAT UPON DIPHTHERIA TOXOID-ANTITOXIN FLOCCULES

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IT was shown by Glenny and Pope (1927) that the antigenic efficiency of toxoid-antitoxin floccules, as measured by the Immunity Index method, was improved by heating for 1 hour at 80° C. Glenny and Waddington (1928) confirmed this observation, and showed that the filtrate from a heated batch produced immunity when injected into guinea-pigs. Ramon, Legroux and Schoen (1931) suggested decomposition of the floccules on heating, with destruction of antitoxin, and solution of the more heat-stable toxoid. Watson, Taggart and Shaw (1941) found that the solubility of floccules and the antigenic activity of filtrates increased with increasing pH level at which the heating was performed up to approximately pH 9·0, above which the activity decreased. They tested the filtrates for toxoid content by means of the flocculation test using an enzyme-purified antitoxin; they were unable to obtain flocculation against other sera.

In the experiments now to be described, the floccules under investigation were suspended in buffer solutions; between pH 6·2 and 7·5 sodium phosphate buffers (Sorensen) were used, and above pH 7·5, sodium borate-boric acid buffers (Palitzsh). The range below pH 6·2 was not used, because early experiments showed that no appreciable quantity of toxoid was freed on heating under these conditions. Filtrates from heated samples were tested for free toxoid content by the flocculation test, at least two types of test being made on each sample, as described later. This was done because the nature of the filtrates suggested that unreliable results might be obtained if any undestroyed antitoxin were present. In agreement with the results of Watson, Taggart and Shaw, no flocculation as a rule could be obtained against unconcentrated sera, though in a few cases flocculation occurred at such a point that it was obviously indicating an incorrect value (e.g. more toxoid present than was originally contained in

the floccules); it was always possible, however, to obtain a result by blending the filtrate with a suitable volume of a toxin of known strength, and testing against an unconcentrated serum. Further tests were made against refined antitoxin, both by direct titration and by testing blends. It was sometimes found that the results obtained by different methods were discrepant, or that more than one zone of flocculation existed. When this occurred, a different antitoxin was used in further tests in order to determine which zone represented the true neutral toxoid-antitoxin reaction; in general the method of the blend test was the most satisfactory one.

It is generally assumed that toxoid-antitoxin floccules consist of pure toxoid and antitoxin, but the abnormal flocculation reactions of these filtrates, either in the form of impossible values or more than one zone, suggests one of two possibilities. The first of these is that the floccules actually contain specific components other than toxoid and antitoxin, and the second that the presence of some undestroyed combined antitoxin causes flocculation in multiple proportions under certain conditions. The latter explanation gains some support in subsequent findings. While the testing of filtrates from most batches of heated floccules presented some difficulty, particularly those heated at pH 8·5 and 9·0, it was especially evident in filtrates from floccules prepared from refined antitoxin, which is more heat-stable than unconcentrated antitoxin. With one exception the floccules used in the experiments to be described were standardized in volume so as to contain approximately 120 Lf per ml. of toxoid. The toxoid and antitoxin after flocculation was allowed to stand for 4 weeks, after which the floccules were washed several times with saline and suspended in buffer solutions.

The effect of heating at different temperatures floccules prepared from the same materials in different proportions.

Three batches of floccules were prepared, using one toxoid and one unconcentrated antitoxin. The first batch (1984) contained approximately 25 per cent less antitoxin than the Lf mixture, the second batch (1985) contained toxoid and antitoxin in equivalent proportions, while the third (1986) contained antitoxin in excess to about 25 per cent. Heating was carried out on 100 ml. quantities of suspension of these floccules at 70° C., 75° C. and 80° C. for 1 hour at pH levels ranging from pH 6·17 to pH 9·0. Table I shows the titres of toxoid in Lf doses per ml. found in the filtrates after heating, and it will be seen that in general the higher the temperature the more toxoid was freed, though there are indications that destruction may occur at 80° C. above pH 8·5. The results confirm those of Watson, Taggart and Shaw in so far as the amount of toxoid present increased with increasing pH level, but in contrast to their findings more toxoid was recoverable under the same conditions from floccules prepared using excess toxoid than from floccules containing an excess of antitoxin. In order further to test this point, five batches of floccules were prepared using another toxoid flocculated with different amounts of a refined antitoxin. These batches contained 0·66, 1·0, 1·5, 2·0 and 2·3 units of antitoxin per Lf dose of toxoid. Portions of the floccules were suspended in buffer solutions and heated for 1 hour at 75° C.; the results are shown in Table II. The essential findings in this experiment are the same as those shown in Table I, for the amount of

TABLE I.—*Showing the Amounts of Toxoid Detectable in Filtrates from 3 Batches of Toxoid-Antitoxin Floccules after Heating for 1 hour at Different pH Levels.*

Batch of floccules	1984			1985			1986		
Units of antitoxin per Lf toxoid	0·75			1·0			1·25		
	Temperature.								
	70°	75°	80°	70°	75°	80°	70°	75°	80°
pH.	Toxoid content of filtrates (Lf per ml.).								
6·17	0	3	6	0	0	3	0	0	2
6·55	1	3	8	0	2	6	1	1	2
7·05	3	11	26	1	10	19	1	3	10
7·65	5	15	29	1	14	28	3	8	10
8·00	13	22	30	3	29	30	3	10	18
8·20	28	43	59	4	40	63	4	22	39
8·50	37	53	*ca.*50	29	*ca.*60	—	12	40	44
9·00	60	60	—	44	—	42	27	42	20

TABLE II.—*Showing the Amounts of Toxoid Detectable in Filtrates from 5 Batches of Toxoid-Antitoxin Floccules after Heating for 1 hour at 75° C. at Different pH Levels.*

Batch of floccules	2110	2123	2124	2125	2126
Units of antitoxin per Lf toxoid	0·66	1·0	1·5	2·0	2·5
pH.	Toxoid content of filtrate (Lf per ml.).				
6·55	5	5	1	1	0
7·05	10	8	3	3	1
7·65	19	10	5	3	1
8·0	35	18	5	3	1
8·2	44	16	10	3	1
8·5	50	14	12	6	1
9·0	50	—	—	6	2

toxoid passing into solution at a given pH level decreases as the antitoxic content of the original floccules increased. This is in direct contrast to the results of Watson, Taggart and Shaw, who found that recovery of toxoid was greatest from floccules in which the antitoxin was considerably in excess.

In all experiments, especially at 80° C., many of the suspensions of floccules underwent coagulation ; those suspended in buffers at pH 8·2 or above frequently went into solution after heating for 10 or 15 minutes.

A comparison of the antigenic efficiency of suspensions of heated floccules containing different amounts of toxoid in solution.

Tests for antigenic efficiency were made by means of two equal doses of material injected subcutaneously into groups of 12 or more guinea-pigs at an interval of 28 days. The animals were bled 10 days after the second injection,

and the individual bleedings tested for antitoxic content by the guinea-pig intracutaneous method. Tests were made at approximately 2-fold differences, the lowest value tested being 0·001 unit. In all tables showing antitoxic response of guinea-pigs it has been necessary to record the median value rather than the geometric mean, because in some groups a proportion of the animals failed to produce any detectable antitoxin. The extent of the scatter of the antitoxic values is of importance in giving some indication of the group response to injection, but the figures obtained in these experiments are too extensive for present-day publication, and have therefore been summarized in the form of median values for each group. With increase in dosage or improvement in antigen scatter is reduced, though the relation between dose and response is not nearly so close for toxoid-antitoxin floccules as for other types of antigen such as toxoid or alum-precipitated toxoid.

Table III gives a comparison of the antigenic efficiency of a number of suspensions produced by heating 100 ml. quantities of a batch of floccules for

TABLE III.—*Showing the Immunity Response of Guinea-pigs to 2 Injections each 0·1 ml. of Toxoid-Antitoxin Floccules Heated at Different pH Levels.*

Sample.	pH.	Free toxoid (Lf per ml.).	Median antitoxic value (units per ml.).
1	6·17	0	0·706
2	7·0	10	0·708
3	8·0	29	2·33
4	8·2	40	3·55
5	8·5	60	1·09
6	unheated control		0·0235

1 hour at 75° C. at different pH levels. The floccules used had been prepared by mixing equivalent proportions of toxoid and unconcentrated antitoxin, and the supernatant liquids from the heated samples contained toxoid in solution in amounts ranging from 0 to 60 Lf doses per ml.

All heated samples were considerably better antigens than the unheated floccules, including that heated at pH 6·17, in the filtrate of which no detectable toxoid was present. The antitoxic response was greatest in guinea-pigs injected with the sample containing 40 Lf per ml. of free toxoid, and a titre of 29 Lf gave a better result than one of 60 Lf. It should be pointed out however that many tests using different dosage would be necessary to determine the optimum balance between free toxoid and undissolved floccules. In this experiment no guinea-pig produced less than 0·1 unit of antitoxin except those injected with the unheated control; only 3 out of 14 in this group produced more than 0·1 unit, and 3 failed to reach 0·001 unit. The use of a lower dose might have suggested that suspensions containing less free toxoid were the better antigens, because in general low doses of precipitated antigens are more effective than low doses of soluble toxoids. In unpublished experiments done at these laboratories it has often been found that a reduction of the dose to one-tenth or one-hundredth of that prescribed under the Therapeutic Substances Act for the testing of toxin-antitoxin floccules (T.A.F.) produces but little difference in the antigenic effect.

A further comparison was made using a different batch of floccules in which refined antitoxin was used; the amount of antitoxin was 80 per cent of that required for neutrality. The samples of floccules were heated for 1 hour at 70° C., and the free toxoid titres of the resulting suspensions ranged from 0 to 44 Lf per ml. In the testing of antigenic efficiency the same method was used, but doses of 0·1, 0·01 and 0·001 ml. were injected for each sample. The results are given in Table IV, and show, as in Table III, that all the heated preparations

TABLE IV.—*Showing the Immunity Response of Guinea-pigs to 2 Injections of Toxoid-Antitoxin Floccules Heated at Different pH Levels.*

Sample.	pH.	Free toxoid (Lf per ml.).	Median antitoxic value (units/ml.) of animals injected with—		
			0·001 ml.	0·01 ml.	0·1 ml.
1	6·55	0	0·050	0·141	0·251
2	7·65	2	0·0141	0·174	0·505
3	8·2	12	0·123	0·317	0·133
4	8·5	27	0·500	0·573	0·833
5	9·0	44	0·234	0·537	1·59
6	unheated control		—	0·0020	0·0913

were better antigens than the unheated floccules. In general the antigenic efficiency increased with the amount of free toxoid in solution, but the samples heated at pH 8·5 and 9·0, with 27 and 44 Lf per ml. respectively, appeared equally efficient. The fact that the latter appeared slightly better than the former when a dose of 0·1 was used, but worse when 0·01 was used, if not due to animal variation, may be the result of the relative proportions of dissolved and undissolved material as suggested earlier, or to the physical condition of the remaining floccules favouring greater antigenic efficiency after heating at pH 8·5 than pH 9·0.

Examination of the results with regard to median value and degree of scatter shows that the values obtained using Samples 1, 2 and 3 are sufficiently comparable to be collected together into a group B for each level of dosage; Samples 4 and 5 form another group, C, and the unheated control alone a third group, A. The distribution of values arranged in this manner is presented in Fig. 1, which gives the percentage of the total number of guinea-pigs exceeding certain values (log scale).

A comparison of the curves shows that all become steeper, owing to reduction in scatter, with increasing dosage. As the dose is increased, the difference in antigenic efficiency of the samples also becomes far less apparent. The most interesting facts, however, arise from a comparison of the actual antitoxic values reached by the majority of guinea-pigs in each group. These can be briefly summarized as follows :—

Group A3 (0·1 ml.), 15 guinea-pigs; 13·3 per cent failed to produce antitoxin, but of the remainder, 73 per cent produced 0·04, 0·1 or 0·2 unit.
Group B3, 39 guinea-pigs, no failures; 79 per cent produced 0·1, 0·2, or 0·5 unit.
Group C3, 25 guinea-pigs, no failures; 78 per cent produced 0·5, 1, or 2 units.

There is but little scatter in groups B and C at this dosage, and the range of values within which the majority fall is somewhat higher for C than B. The

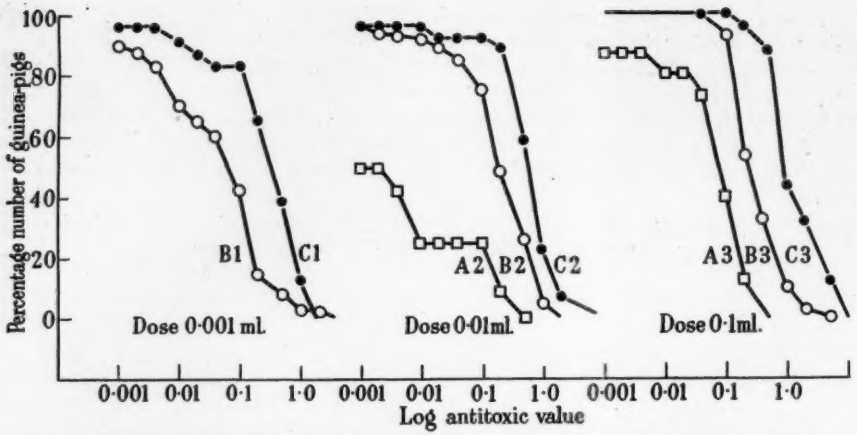

FIG. 1.—Showing the percentage number of guinea-pigs exceeding certain antitoxic values after two injections of unheated toxin-antitoxin floccules (Curves A), heated toxin-antitoxin floccules Samples 1 to 3 in Table IV (Curves B), and Samples 4 and 5 (Curves C).

chief difference between A and B lies in the failure of 13 per cent of group A animals to respond ; the majority of the responders produced values comparable with the majority of group B animals.

Decreasing the dose to 0·01 ml., the difference between A and the other groups is more apparent : 50 per cent of the 12 guinea-pigs (A2) failed to respond, and 33 per cent of the remainder had values of 0·1, 0·2 unit. Group B2, 65 guinea-pigs, 3·1 per cent failures, 71 per cent of the responders produced 0·1, 0·2, 0·5 unit. Group C2, 28 guinea-pigs, 3·6 per cent failures, 82 per cent of responders produced 0·2, 0·5, 1 unit. At this level of dosage, therefore, A is inferior to B and C for two reasons ; there is a much larger percentage of non-responders, and a much smaller group with values equal to the majority of responders in B and C.

Using the lowest dose (0·001 ml.) the control sample A was not tested. In Group B1, 10·3 per cent of the 39 guinea-pigs failed to respond, and 59 per cent of the remainder had values of 0·04, 0·1, 0·2 units, while in Group C1, 4·4 per cent of 23 guinea-pigs failed, and 69 per cent of the responders produced 0·1, 0·2, 0·5 units.

These results show that the majority of those guinea-pigs which showed an antitoxic response produced values which fall within a small range, and this range scarcely altered even when the dose injected was increased or decreased 10 to 100-fold.

In an attempt to determine the cause of the improvement in antigenic efficiency of heated samples whose filtrates contain no detectable toxoid, one such batch was centrifuged, the supernatant liquid removed, the floccules well washed several times with saline and resuspended to original volume. This suspension and the unwashed heated suspension were injected into guinea-pigs. When two doses each of 0·1 ml. were injected, there was no difference in the response of the two groups of animals, but using a dose of 0·01 ml. the unwashed

suspension appeared to be slightly better; both preparations were superior to the parent unheated floccules. A single injection of 5·0 ml. of the supernatant liquid produced low-grade immunity in another group of guinea-pigs, 8 out of 13 animals producing from 0·001 to 0·2 unit of antitoxin in 6 weeks, and the remaining 5 failing to respond. It appears probable that the increased antigenic efficiency of preparations containing no detectable toxoid is due to some physical change in the floccules during heating, as well as to the action of traces of toxoid undetectable by blend flocculation test, or of soluble toxoid-antitoxin. Support is lent to this idea by the results of the following experiment.

The effect on antigenic efficiency of addition of heated dissolved toxoid-antitoxin floccules to unheated floccules.

As has been stated previously, batches of floccules heated at 75° C. or 80° C. at pH 8·5 or 9·0 usually pass completely into solution. It appeared possible that the properties of heated floccule suspension might be reproduced by the addition of heated dissolved floccules to an unheated suspension. A batch of floccules was therefore heated at 75° C. for 1 hour at pH 8·5, and the resulting solution found to contain 60 Lf doses of toxoid per ml. Portions of this solution were then added to measured volumes of a batch of unheated floccules in such quantities that the final concentration of toxoid in the mixtures was 5, 10, 20, 30 and 45 Lf per ml.

All these mixtures therefore contained the same total nitrogen and the same amount of total antigen, though the proportions of floccules to dissolved floccules varied.

The antigenic efficiency of the mixtures was tested by the usual method of two injections into guinea-pigs, and the results are given in Table V. These results should be compared with those in Table IV, because the batch of floccules

TABLE V.—*Showing the Immunity Response of Guinea-pigs to 2 Injections of Toxoid-Antitoxin Floccules to which Dissolved Floccules had been Added.*

Sample.	Toxoid added (Lf per ml.).	Median antitoxic value (units/ml.) of animals injected with—	
		0·001 ml.	0·01 ml.
1	5	Under 0·001	0·0089
2	10	0·050	0·040
3	20	0·0036	0·034
4	30	0·016	0·063
5	45	0·080	0·040

used was the same for both experiments. The mixtures were all better antigens than the parent batch (unheated control, in Table IV), but none was nearly so efficient as any of the heated samples in Table IV; some failures to respond occurred in every group. This result strongly suggests that increased antigenic power due to heating is due not only to destruction of antitoxin with liberation of soluble toxoid, but also to some change in the remaining floccules, probably either in solubility in the tissues, stability or ease of dissociation.

The effect of phenol in the heating of toxoid-antitoxin floccules.

In a single experiment a batch of floccules prepared from refined antitoxin was divided into four portions treated as follows :—

(*a*) Suspended in buffer at pH 7·1, no phenol used
(*b*) „ „ „ pH 8·0, „ „ „
(*c*) „ „ „ pH 7·1, 0·5% phenol added
(*d*) „ „ „ pH 8·0, 0·5% „ „

These suspensions were heated for 1 hour at 80° C. and tested for antigenic efficiency. Portions of the filtrates were tested for toxoid content by the flocculation test, and it was found that within the limits of error of the test the same titre was obtained in the presence or absence of phenol for both pairs of suspensions. In the antigenic efficiency test, using 15 guinea-pigs for each sample, the presence of phenol during heating had no apparent effect at pH 7·1, but at pH 8·0 the sample containing phenol was definitely inferior to the control heated without phenol. It might well be expected that denaturation of toxoid would occur at pH 8·0 or higher in the presence of phenol, but further work is necessary before any final conclusion can be drawn.

The effect of salt.

It is well known that toxoid-antitoxin floccules show a certain degree of solubility in distilled water, but appear to be relatively insoluble in normal saline solution ; they also go into solution at low pH levels (Glenny and Pope, 1927). In order to facilitate recovery of toxoid by heat-treatment, reduction of the salt content of the suspending liquid should presumably be aimed at, in order to increase solubility, although at certain pH levels (above about pH 8·5) complete solution may readily occur on heating. If distilled-water suspensions of floccules are heated in glass containers, changes of pH are very liable to occur, and the conditions cannot therefore be controlled. Because of this, floccules used in the experiments described here have been suspended in buffer solutions free from sodium chloride. The pH level has thus been stabilized at the cost

TABLE VI.—*Showing the Effect on Toxoid Liberation of the Addition of Sodium Chloride to Floccules Suspended in Buffer Solutions and Heated at 80° C.*

pH.	Toxoid content of filtrates (Lf/ml.) recovered from floccules.					
	A.		B.		C.	
	With NaCl.	No NaCl.	With NaCl.	No NaCl.	With NaCl.	No NaCl.
6·55	10	18	6	6	2	3
7·05	22	40	10	8	2	4
7·65	31	50	14	15	3	5
8·0	30	52	16	31	4	6
8·2	53	110	26	55	7	9
8·5	30	120	21	50	8	16
9·0	—	*ca.*30	10	10	6	5
Units of antitoxin per Lf toxoid in floccules.	0·66		1·0		1·5	

of the inclusion of certain electrolytes. The effect of the addition of sodium chloride to the buffers has, however, been studied. In these experiments portions of a batch of floccules were freed from salt by washing with distilled water, and suspended in buffer solutions; the companion sets were suspended in saline solution, which was drawn off and the floccules suspended in buffers containing 0·20 per cent sodium chloride. Three batches of floccules were used, one, A, in which 0·66 equivalent of antitoxin was present, the second batch, B, one equivalent, and the third, C, 1·5 units of antitoxin per Lf dose of toxoid. The results of heating these suspensions are shown in Table VI. The presence of sodium chloride strongly inhibits the liberation of toxoid from the floccules, and in many instances the titre of toxoid found in the sodium chloride samples is of the order of half that in the corresponding controls.

Total nitrogen estimations were done on these filtrates for the purpose of comparing the purity of the free toxoid in different series. In every case the purest filtrates were obtained at pH 8·2, and the actual figures for purity of these samples in terms of Lf per mg. nitrogen are as follows :—

	Lf with sodium chloride.	Lf without sodium chloride.
Batch A	600	790
„ B	330	510
„ C	127	145

These figures show that the filtrates contain considerable amounts of soluble protein derived from the antitoxin constituent, because the purities of filtrates from Batch C are low and even those from Batch A are not especially high; the more antitoxin was used in the preparation of the floccules, the less pure the resulting filtrates after heating, in terms of Lf doses per mg. N. This would obviously be expected if the antitoxin-nitrogen were not coagulated during heating.

It was noteworthy that the presence of sodium chloride increased the tendency to coagulation of floccules on heating, and no samples heated under these conditions dissolved completely. Whether these effects are due to sodium chloride itself or to the increase in the total ionic concentration of the suspending solution has not been investigated.

The effect of time of heating upon antigenic efficiency and toxoid liberation.

In view of the fact that floccules heated under conditions favouring their complete solution dissolve in a very short time (10–15 minutes), it appeared possible that any changes that occur might take place very quickly, and that prolonged heating might either be unnecessary or produce a detrimental effect. Experiments were therefore carried out to determine the optimum time of heating to liberate maximum amounts of toxoid, and to produce the best type of antigen. Table VII shows the effect of time of heating of two batches upon the amount of toxoid passing into solution at different pH levels. In some instances, e.g. Samples 4 and 6, time has but little effect upon toxoid liberation, and usually the maximum was reached in an hour. In all experiments involving Batch Y, destruction of toxoid occurred with longer heating; with Batch X, destruction occurred only at pH 8·5 between 1 and 2 hours. Unless this were due to individual batch-variation, it appears possible that splitting of floccules made from refined

TABLE VII.—*Showing the Amounts of Free Toxoid Detectable in Filtrates from Toxoid-Antitoxin Floccules after Heating for Different Lengths of Time.*

Sample.	Batch of floccules.	Type of antitoxin in floccules.	pH.	Temperature.	Lf per ml. in filtrate after heating for—			
					15	30	60	120 min.
1	X	Unconcentrated	7·05	75° C.	6	12	11	13
2	X	,,	8·0	75° C.	16	24	28	28
3	X	,,	8·5	75° C.	45	49	72	54
4	Y*	Refined	7·05	75° C.	5	5	6	4
5	Y	,,	8·0	75° C.	14	13	19	9
6	Y	,,	7·05	80° C.	9	10	7	7

* This batch contained only 80 Lf per ml.

antitoxin occurs more rapidly than splitting of those from unconcentrated antitoxin, and that the toxoid thus liberated is somewhat more stable to heat in the latter case than the former. Presumably both rate of splitting and subsequent stability are to some extent dependent upon the firmness of union of the floccule components. Solubility of floccules may also be dependent upon firmness of union, for it has repeatedly been noticed in these laboratories that non-avid antitoxins (i.e., those combining loosely with toxin) give a very meagre yield of floccules, suggesting that all the toxoid-antitoxin does not come out of solution. It is possible that floccules from refined antitoxin are more readily soluble than those from unconcentrated antitoxin, though this has not been shown, and in their behaviour on heating at pH 8·5 or above there is no observable difference.

If the antigenic efficiency of heated preparations were related entirely to their free toxoid content, it would be expected that time of heating would have but little effect, with most of the preparations shown in Table VII. That this is in general not the case is shown in Table VIII, which deals with most of the

TABLE VIII.—*Showing the Immunity Response of Guinea-pigs to 2 Injections of Some of the Suspension Featured in Table VII.*

Sample in Table VII.	Dose injected.	Median antitoxic value of guinea-pigs injected with samples heated—			
		15	30	60	120 min.
1	0·01	—	0·163	—	0·163
3	0·0067	0·100	0·130	0·0063	0·0040
4	0·01	0·0470	0·141	< 0·001	< 0·001
5	0·01	0·014	< 0·001	< 0·001	< 0·001
6	0·01	0·163	0·130	< 0·001	< 0·001

samples shown in Table VII. The results show that the antigenic efficiency of Samples 4, 5 and 6 was severely damaged if heating was maintained for as long as 1 hour, although the free toxoid content might increase between 30 minutes and 1 hour. Using Samples 1 and 3 (from the first batch of floccules) this was not so apparent, though with Sample 3 a considerable reduction in antigenic efficiency occurred between 30 minutes and 1 hour and 2 hours, despite the fact that the 1-hour sample contained a much higher titre of toxoid than the other samples. No difference in efficiency was obtained in the case of Sample 1,

whether heated for half an hour or two hours. It should be noted, however, that no guinea-pigs injected with these samples gave a poor response, and it is possible that the use of lower dosage might have revealed differences.

It is apparent that the majority of suspensions reach their maximum antigenic efficiency after heating for 30 minutes or less. Longer heating, though this may liberate more toxoid, damages the antigenic properties, in some cases severely. This effect is thought to be due to denaturation of toxoid in solution, rather than to any effect on the remaining floccules, since Sample 3 in Table VIII became completely dissolved after 10 minutes' heating, so that all the samples in this series were clear solutions.

These experiments show that the amount of toxoid passing into solution on heating under given conditions of pH and temperature is dependent upon the composition of the floccules heated ; that, in general, increase of pH or temperature causes liberation of greater amounts of toxoid. The addition of relatively small concentrations of sodium chloride to suspending buffers may make an enormous difference to the heated product, and time of heating has a very significant, but not constant, effect upon the antigenic efficiency and toxoid content of the resulting suspension. Other experiments, not described here, have shown that batches of floccules of the same composition in terms of toxoid : antitoxin ratio, but containing different toxoids, do not behave in the same manner when heated under apparently identical conditions ; it is not possible to predict, *a priori*, the yield of soluble toxoid under any given conditions, nor whether coagulation will occur. This may be due to the presence of traces of certain inorganic salts in some batches. Further, relatively large amounts of antitoxic protein are present in most filtrates, and it is surprising that Watson, Taggart and Shaw describe their filtrates as " highly " concentrated and purified antigens " containing mere traces of dissolved matter." The purest filtrate obtained in my experiments contained less than 800 Lf per mg. of nitrogen. The experiments also suggest that some of the material present in the filtrates exists as loosely-combined undestroyed antitoxin ; this has actually been demonstrated by means of the guinea-pig intracutaneous test. The addition of toxin to a filtrate has turned out of combination some of the toxoid, thereby sensibly reducing the toxicity of the toxin through combination with traces of freed antitoxin. In these same filtrates the presence of toxoid was demonstrated by a quantitative flocculation test. The nature of the filtrates is therefore not simple, but appears to consist of toxoid, under certain conditions in a somewhat denatured state, in loose combination with antitoxin. The quantitative relation between the toxoid and antitoxin cannot be worked out by any simple means, but it is probable that the toxoid is grossly in excess. It is also possible in such a system that combination in multiple proportions may occur.

SUMMARY.

When diphtheria toxoid-antitoxin floccules are heated, more toxoid is recovered under the same conditions of pH and temperature from underneutralized floccules containing less than one equivalent of antitoxin than from those prepared with one equivalent or more. The amount of toxoid released decreases as the proportion of antitoxin in the floccules increases. Suspensions heated above pH 8·2 usually dissolved completely.

The antigenic efficiency of floccules heated under conditions so as to recover varying amounts of toxoid increases with the amount of toxoid released, appearing to be maximal when 30–40 Lf/ml. are present in solution, but decreases again at a concentration of 60 Lf/ml. The original floccules contained 120 Lf/ml. of toxoid.

The antigenic efficiency of unheated floccules plus heated floccules solution is inferior to that of heated floccules, suggesting that heating brings about changes in the floccules other than dissociation, and destruction of antitoxin.

The presence of sodium chloride in suspending buffer inhibits liberation of toxoid on heating. This may, however, be due to the increase in the total ionic concentration.

The purest filtrate obtained contained 790 Lf doses per mg. nitrogen, showing that considerable quantities of soluble antitoxic protein were present. This was obtained from floccules of composition 1 Toxoid: 0·67 antitoxin: purities of filtrates from 1:1 or 1:1·5 floccules were considerably lower.

The antigenic efficiency of suspension of floccules increases on heating for 15 to 30 minutes, after which it is usually severely damaged, even if no destruction of soluble toxoid occurs.

The results suggest that filtrates from heated floccules consist of relatively small amounts of antitoxin loosely combined with and grossly over-neutralized by toxoid.

I am indebted to Mr. A. T. Glenny, F.R.S., for much helpful criticism, and to Miss Llewellyn-Jones and testing staff for much technical assistance in the animal tests.

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THE LEWIS BLOOD GROUPS OF 79 FAMILIES.

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THE "Lewis" blood groups were discovered in 1946 by Mourant, who found a "new" antibody in two samples of human serum. This antibody, which was called anti-Lewis, agglutinated the red blood cells of 24 out of 96 English people of group O. The antigen thus recognized appeared to be independent of the ABO, MN, Rh, P and Lutheran antigens.

The antibody gave reactions slightly stronger at room temperature than at 37° C.; both examples were considered to be spontaneous rather than immune.

Mourant tested the families of 15 persons who were Lewis positive. In 7 cases both parents of a Lewis-positive person were examined, and one or the other parent was also Lewis positive in all of them. Mourant said, "This is conclusive evidence that the agglutinogen is inherited, and suggestive, but not statistically significant evidence that it is a Mendelian dominant character rather than a recessive." Considering that all other known blood group genes express themselves in single dose this restraint was most praiseworthy, and justified in the light of subsequent investigations.

More examples of anti-Lewis sera were quickly found; a description of one was published by Jakobowicz, Simmons and Bryce (1947). Several were sent to Dr. Mourant, at the Blood Group Reference Laboratory, from the English and Welsh Blood Transfusion Services. An example was found by Dr. Hartmann in Oslo, and one of us (R. S.) found 4 examples in a collection of antisera set aside for her investigation by Dr. L. K. Diamond, at the Blood Grouping Laboratory, Boston.

In 1947 Andresen reported that he and Freiesleben, working independently, had found, during the course of the year, 8 examples of an antiserum which agglutinated the red cells of 21 per cent of adults. Andresen (1947) called this blood group system the L system, which was in a way fortunate, for it proved subsequently to be the Lewis system of Mourant. Andresen's choice of a symbol was in another way less fortunate, for there was at the time in the English press a paper by Mainwaring and Pickles (1948) describing 3 examples of the anti-Lutheran antibody of Callender and Race (1946), and defining subdivisions of the Lutheran groups for which Mainwaring and Pickles used the symbols L_1, L_2 and l.

Andresen's paper considerably advanced knowledge of the Lewis blood groups. The frequency of Lewis-positive blood in adult Danes was established as 21 per cent, and the unexpected observation was made that the frequency was higher in infants. These points are illustrated in Table I, which is taken from

TABLE I.—*Type Distribution Demonstrated in Adults and in Children at Different Ages (from Andresen).*

Type.	Adults.		Children.							
			0–3 months.		4–6 months.		7–9 months.		10–12 months.	
	No.	%	No.	%	No.	%	No.	%	No.	%
L+	166	21	78	79	74	73	18	36	19	29
L−	618	79	21	21	28	27	32	64	46	71

Andresen's paper. Andresen found, moreover, that " parents of type L− very well might get children of type L+," and in order to satisfy these two sets of observations proposed that, while in adults only LL homozygotes gave the reaction L+, in young infants on the other hand the heterozygotes Ll were also giving the L+ reaction. In other words, in adults the Lewis+ group was behaving as a recessive Mendelian character. This was unexpected, for perhaps all other known blood group genes express themselves when in single dose. One of the main purposes of this present paper is to give evidence which appears strongly to support Andresen's view that Lewis-positive adults are genetically homozygous.

In 1948 Andresen published a second paper which again advanced the subject. Andresen reported the finding of another type of antiserum (anti-L_2), whose reactions bore an almost completely antithetical relationship to those of the original anti-Lewis (called by Andresen in this second paper anti-L_1). Table II, which is taken from the paper referred to, shows the result of testing 238 group O blood samples (evidently from adults) with the two sera.

TABLE II.—*Distribution of the Groups in the L System within Group O (from Andresen).*

	$L_1+ L_2+$.	$L_1+ L_2-$.	$L_1- L_2+$.	$L_1- L_2-$.	Total.
Group O	0	46 (20%)	178 (74%)	14 (6%)	238

As Andresen pointed out, these results fit very well with the concept that L_1 and L_2 are allelomorphic antigens, but that the existence of the small L_1- L_2- group made it " not yet possible to establish the manner of inheritance with certainty."

Such were the results obtained by Andresen with adult group O cells and also with group A_2 cells. When A_1 cells were tested, however, it was found that only 42 per cent were agglutinated by the anti-L_2 serum, and that many of the reactions were weak or doubtful. Andresen considered this effect to be an example of the phenomenon known in genetics as epistasis, L_2 being suppressed if A_1 were also present. Epistasis is defined by Waddington as follows: " Another form of interaction between multiple factors is found when one factor is ' dominant ' over the others, i.e. suppresses their expression. We cannot use the terms dominance and recessiveness in such cases since the factors involved occupy different loci and are not allelomorphs. Bateson proposed the words epistasis and hypostasis." Andresen found that in children the percentage of group O bloods agglutinated by the anti-L_2 serum was considerably lower than in adults.

In July, 1948, Dr. Andresen visited England and kindly demonstrated the reactions of the anti-L_2 serum. Later we recognized a second example of anti-L_2 in a serum sent to us by Dr. Peter Vogel and Dr. R. E. Rosenfield of New York.

In October, 1948, Grubb, working at the Lister Institute, made an observation which greatly added to the interest of these many-sided groups, and which started a fresh line of investigation. Grubb discovered that practically all, if not all, persons whose red cells were Lewis positive (L_1+) were also salivary non-secretors of A, B or H substance, and *vice versa*.

Early in 1949 certain people engaged in work on the Lewis groups (Andresen, Callender, Fisher, Grubb, Morgan, Mourant, Pickles and Race, 1949) agreed to use the following notation for the Lewis and Lutheran systems.

System	Lewis	Lutheran
Genes	Le^a	Lu^a
	Le^b	Lu^b
Genotypes	Le^aLe^a	Lu^aLu^a
	Le^aLe^b	Lu^aLu^b
	Le^bLe^b	Lu^bLu^b
Phenotypes	Le(a + b −)	Lu(a +)
	Le(a − b +)	Lu(a −)
	Le(a − b −)	
Antibodies	anti-Le^a	anti-Lu^a
	anti-Le^b	

No distinction is made between the symbols for the genes and those for the antigens.

The antibody anti-Le^a corresponds to the original (Mourant, 1946) anti-Lewis and to the anti-L_1 of Andresen, anti-Le^b corresponds to the anti-L_2 of Andresen (1948).

If only one anti-serum, anti-Le^a, is used the phenotypes which it defines are called Le(a +) and Le(a −).

This notation will be used in what is to follow.

EXPERIMENTAL.

Samples of blood from 571 unrelated persons have been tested with anti-Le^a; 130 or 22·77 per cent were agglutinated. Table III shows the samples divided

TABLE III.—*Lewis Blood Groups of* 571 *Unrelated Persons.*

	O.	A_1.	A_2.	B.	A_1B.	A_2B.	$A_1 : A_2$ undifferentiated. A.	$A_1 : A_2$ undifferentiated. AB.	Total.
Le(a+)	59	47	9	9	2	1	3	0	130
	21·93%	25·68%	18·37%	21·95%					22·77%
Le(a−)	210	136	40	32	6	6	10	1	441
									77·23%
Total	269	183	49	41	8	7	13	1	571

according to the A_1 A_2 B O blood groups. In view of the influence of the A_1 antigen on the reaction of anti-Le^b serum the following comparison has been made :

	Le(a +).	Le(a −).	Le(a +) %.
Samples containing A_1 . .	49	142	25·65
Samples without A_1 . . .	78	288	21·31

But as $\chi^2 = 1{\cdot}3$ for one degree of freedom, the excess of Le(a +) blood samples which contain A_1 can well be accounted for by chance, and it can be assumed that the reaction of a sample of blood with anti-Le^a is independent of its A_1 A_2 B O group.

Of the 571 blood samples 406 were from English people ; 132 were from American whites, tested in Boston by one of us (R. S.), and 33 were from people known to be Welsh.

Although the exact manner of inheritance of the Lewis groups as defined by anti-Le^a and anti-Le^b is not yet established with certainty, the following considerations, confined to the results given by the original anti-Lewis (anti-Le^a) type of serum, strongly support Andresen's theory that the presence of the Le^a antigen is in adults a recessive character.

If Le(a +) blood is genetically homozygous (Le^aLe^a), then the gene frequency of $Le^a = \sqrt{0{\cdot}2277} = 0{\cdot}4772$ and that of the other allelomorphs $= 1 - 0{\cdot}4772 = 0{\cdot}5228$. Representing the other allelomorphs by Le? the expected genotype frequencies would then be :

Le(a +)	Le^aLe^a	0·2277
Le(a −)	$Le^aLe^?$	0·4990
	$Le^?Le^?$	0·2733

and these figures can be applied to the examination of the results of testing the 79 families given in Table IV.

The families have been tested for other blood group systems than those shown in the table, but the addition of these groups would make the table too involved for the present purpose. All the children are over the age of 2 years.

Table V shows the children expected from the various types of mating, on the assumption that Le(a +) blood is homozygous.

From Table V it can be calculated that the children from a mating Le(a +) × Le(a −) should be in the ratio of 0·3230 Le(a +) to 0·6770 Le(a −). From the mating Le(a −) × Le(a −) the children should be in the ratio of 0·1044 Le(a +) to 0·8956 (Le(a −). From the mating Le(a +) × Le(a +) all children would be expected to be Le(a +).

Table VI shows the comparison between the children expected and those observed in the 79 families. The agreement is seen to be very close ; and this strongly supports the theory of Andresen.

As a contrast Table VII shows the observed children from the various matings compared with the expected children, but with the expectations this time based on the obvious alternative possibility that Le(a +) blood may be either homozygous or heterozygous for Le^a. The following gene frequencies have been used in the calculation of the children expected.

$$Le^? = \sqrt{0{\cdot}7723} = 0{\cdot}8788 \text{ and } Le^a = 1 - 0{\cdot}8788 = 0{\cdot}1212.$$

TABLE IV.—*The Lewis and the A_1 A_2 B O Groups of 79 Families, some of them also Classified for A, B and H Secretion.*

Matings Le(a −) × Le(a +).

Family No.	Father. A_1 A_2 BO	Le(a)	Mother. A_1 A_2 BO	Le(a)	Children. Le(a −).	Le(a +).
1	A_1	+	A_2	−	O	$A_2.A_2$
2	A_1 B	+	O	−	B	..
3	A_1	−	O	+	..	O.A_1
4	A_1	+	A_1	−	$A_1.A_1$	..
	ss		S		S S	
5	O	−	A_1	+	A_1	A_1
6	A_1	+	O	−	A_1	$A_1.A_1$
7	A_1	+	A_1	−	A_1	$A_1.A_1$
	ss		S		S	ss ss
8	O	+	A_1	−	..	A_1
9	O	+	O	−	O.O.O	..
10	A_1	−	A_1	+	..	$A_1.A_1$
11	O	−	A_1	+	..	$A_1.A_1$.O
						ss
12	A_1	+	A_1	−	A_1	A_1
13	A_1	−	A_1	+	O	A_1.O.O
14	A_1	+	B	−	A_1 B.B	..
15	O	+	A_1 B	−	B.B	..
16	A_1 B	+	O	−	A_1.B.B.A_1.A_1.B	..
	ss		S		S S S S S	
17	O	+	O	−	O.O	..
18	O	−	O	+	O.O	..
19	A_1	−	B	+	..	$A_2.A_1$ B.A_2 B
20	O	+	O	−	O.O.O	..
21	A_2	−	O	+	O	..
					S	
22	A_2	+	A_1	−	$A_2.A_1$	..
					S	
23	O	+	B	−	B	O
	ss					
24	A_1	−	A_1	+	A_1	..
25	A_1	−	O	+	A_1.O	..
	S		ss		S	
26	A_2	+	O	−	O	..
					S	
27	O	+	O	−	O.O.O	O
	ss		S		S S S	ss
28	O	−	O	+	O.O	..
	S		ss		S S	
29	A_1	+	O	−	..	A_1
	ss		S			ss
30	B	−	O	+	O	B.O
	S		ss		S	ss ss
31	O	+	A_2	−	A_2.O.O	..
	ss		S		S S S	

TABLE IV (*continued*).

Matings Le(a −) × Le(a +).

Family No.	Father. A1 A1BO Le(a)		Mother. A1 A1BO Le(a)		Children. Le(a −).	Le(a +).
32	A_1 ss	+	A_1 S	−	..	A_1 ss
33	A_1 ss	+	O S	−	$A_1.A_1$ S S	..
34	A_2 ss	+	A_1 S	−	$A_2.A_1$ S S	A_1 ss
35	O ss	+	O S	−	..	O ss

Matings Le(a −) × Le(a −).

36	A_1	−	O	−	O	O.O
37	B S	−	B S	−	B.O S	..
38	O S	−	O S	−	O.O	..
39	A_1	−	O S	−	..	O
40	A_1	−	A_1 S	−	$O.A_1.A_1$ S	..
41	O	−	A_1	−	..	O
42	A_1 S	−	O S	−	O S	..
43	B	−	O	−	O	..
44	A_1	−	A_2	−	O	..
45	O	−	A_1	−	..	O
46	O	−	O	−	O.O.O	..
47	O	−	A_1	−	$O.A_1$	..
48	A_1	−	A_2 B	−	A_2 B.A_1	..
49	O S	−	O S	−	O.O S	..
50	O	−	B	−	B.O.O.B.B.O	..
51	A_1 S	−	A_1 S	−	$A_1.A_1.O$ S S	..
52	O	−	A_1	−	$A_1.O.A_1$	..
53	O S	−	O	−	O.O.O	..
54	O	−	A_1	−	$O.A_1$	..
55	A_1 B S	−	O S	−	B S	..
56	O	−	O	−	O.O	..
57	A_1 B S	−	O S	−	B.$A_1.A_1$ S	..
58	A_1 S	−	O S	−	$A_1.A_1.O$ S S	A_1 ss

TABLE IV (*continued*).

Matings Le(a −) × Le(a −).

Family No.	Father. A1A2BO	Le(a −)	Mother. A1A2BO	Le(a −)	Children. Le(a −)	Le(a +)
59	A1	−	B	−	..	O
	S		S			ss
60	O	−	O	−	O.O.O	..
	S		S		S S	
61	O	−	A2	−	A2.A2	..
	S		S		S S	
62	A1	−	A1	−	A1.A1.A1	..
	S		S		S S S	
63	O	−	A1	−	O	..
	S		S		S	
64	O	−	A2	−	O.O.A2	..
	S		S		S S S	
65	A2	−	O	−	A2.A2	O
	S		S		S S	ss
66	A1	−	A1	−	O	..
	S		S		S	
67	A1	−	A1	−	A1	..
	S		S		S	
68	O	−	A2	−	A2.O	..
	S		S		S S	
69	O	−	O	−	O.O.O.O	O
	S		S		S S S S	ss
70	A1	−	A1	−	A1.A1	..
	S		ss			
71	B	−	A1	−	A1 B	..
	S		S		S	
72	A1 B	−	O	−	A1.B	..
	S		S		S S	
73	A	−	O	−	O	..
	S		S		S	
74	B	−	B	−	B	..
	S		S			

Matings Le(a +) × Le(a +).

75	O	+	A2	+	..	A2.A2
	ss		ss			ss ss
76	B	+	O	+	..	O
77	O	+	O	+	..	O.O.O.O
	ss		ss			ss ss ss ss
78	B	+	A1	+	..	A1 B. O.O
	ss		ss			ss ss ss
79	O	+	O	+	..	O.O.O
	ss		ss			ss ss ss

The fathers of Families 55 and 57 are brothers.
The mother of Family 76 is the child in Family 45.

S = salivary secretor of A, B or H.
ss = salivary non-secretor of A, B or H.

TABLE V.—*Showing the Expected Group Frequencies of the Children from Different Lewis Matings. The Expectations are Based on the Theory of Andresen that Le(a +) Blood is Genetically Homozygous.*

	Expected frequency of mating.	Expected children.		
		Le^aLe^a.	$Le^aLe^?$.	$Le^?Le^?$.
$Le^aLe^a \times Le^aLe^a$	0·0518	0·0518	..	..
$Le^aLe^a \times Le^aLe^?$	0·2272	0·1136	0·1136	..
$Le^aLe^a \times Le^?Le^?$	0·1245	..	0·1245	..
$Le^aLe^? \times Le^aLe^?$	0·2490	0·0623	0·1245	0·0623
$Le^aLe^? \times Le^?Le^?$	0·2728	..	0·1364	0·1364
$Le^?Le^? \times Le^?Le^?$	0·0747	..	..	0·0747
	1·0000	0·2277	0·4990	0·2734

TABLE VI.—*The Expected and the Observed Absolute Numbers of Children from the Three Types of Lewis Matings. The Expectations being Based on the Theory of Andresen.*

Mating.		Children.					
Type.	Number.	Number obs.	Le(a +).		Le(a −).		χ^2 for 1 d.f.
			Exp.	obs.	Exp.	obs.	
Le(a +) × Le(a +)	5	13	13	13	0	0	..
Le(a +) × Le(a −)	35	80	25·8	30	54·2	50	0·99
Le(a −) × Le(a −)	39	84	8·8	9	75·2	75	0·01

TABLE VII.—*The Expected and the Observed Absolute Numbers of Children from the Three Types of Mating. The Expectations are Based on the Assumption, no longer held, that Le(a +) Blood may be Genetically either Homozygous or Heterozygous.*

Mating.		Children.					
Type.	Number.	Number obs.	Le(a +).		Le(a −).		χ^2 for 1 d.f.
			Exp.	Obs.	Exp.	Obs.	
Le(a +) × Le(a +)	5	13	10·2	13	2·8	0	..
Le(a +) × Le(a −)	35	80	42·6	30	37·4	50	7·95
Le(a −) × Le(a −)	39	84	0·0	9	84·0	75	..

It will be seen from Table VI that the results of testing the blood of 79 families for the two groups, Le(a +) and Le(a −), are in good agreement with the contention of Andresen that adult Le(a +) blood is genetically homozygous.

Table VII shows that the results contradict the alternative possibility that Le(a +) blood may be either homozygous or heterozygous for Le^a.

It may be wise to avoid speaking of the Le^a antigen as behaving in adults as a Mendelian recessive character and therefore differing from other blood group antigens. Most anti-Lewis sera are of low titre, and it may be that if more powerful sera were available the Le^a gene would be recognizable in single

dose. The appearance of recessivity may be due to the weakness of the antisera at present available.

Samples of saliva from 86 of the parents and 86 of the children shown in Table IV have been tested for the presence of the A, B or H substances. It will be seen below that the results are in accordance with the observation of Grubb (1948) that all people so far tested whose red cells are Le(a +) are salivary non-secretors of the A, B and H substances, and that with a very few exceptions people whose red cells are Le(a −) are salivary secretors of the A, B and H substances.

The following figures have been counted from Table IV :

		Red cells.	
		Le(a +).	Le(a −).
A, B or H in saliva	Secretors . . .	0	123
	Non-secretors . . .	48	1

The results of the family groupings have been counted in a number of different ways in a search for relationships that might possibly exist. The most striking of these counts was the following, taken from the Le(a +) × Le(a −) matings :

	Children.	
	Le(a +).	Le(a −).
Mothers Le(a +) . .	16	11
Mothers Le(a −) . .	14	39

and for this 2 × 2 table $\chi^2 = 8{\cdot}2$, which corresponds to a probability of less than 1 in 400. The main upset in the table is due to too many of the children of Le(a +) mothers being themselves Le(a +).

In attempting to decide whether this disturbance has some cause other than chance it must be remembered that the data provided by the families have been counted in about 20 different ways, and this must lower somewhat the level of probability which is to be considered statistically significant.

Subdivisions of the count suggest that the disturbance, low as the probability is of it being a chance one, nevertheless may be due to chance. If the first 16 families and the last 19 families are counted separately the total number of children in each count is about the same, but the association of Le(a +) mothers and Le(a +) children is found only in the first count.

First 16 *Families.*

	Children.	
	Le(a +).	Le(a −).
Mothers Le(a +) .	11	2
,, Le(a −) .	8	20

Last 19 *Families.*

	Children.	
	Le(a +).	Le(a −).
Mothers Le(a +) .	5	9
,, Le(a −) .	6	19

Had there been a real association between the Le(a +) mothers and Le(a −) children the disturbance would have been expected to show in both counts.

Further, if a count is made omitting all the families with only Le(a −) children, as possibly having a homozygous negative parent, the following figures are obtained :

	Children.	
	Le(a +).	Le(a −).
Mothers Le(a +) . .	16	3
,, Le(a −) . .	14	10

As $\chi^2 = 3{\cdot}4$ for one degree of freedom this is a further indication that the disturbance originally observed may be due to chance.

Two other counts which showed a deviation from the expected proportion will be given in order that attention may be drawn to the deviations and future work show whether they are of significance :

Mating Le(a +) × Le(a −).

	Children.	
	Le(a +).	Le(a −).
Mothers A_1 or A_1 B . . .	15	13
,, O, A_2, B or A_2 B . .	15	37

χ^2 for 1 d.f. = 4·8.

Mating Le(a +) × Le(a −).

	Children.	
	Le(a +).	Le(a −).
Children A_1 and A_1 B . . .	17	16
,, O, A_2, B or A_2 B . .	13	34

χ^2 for 1 d.f. = 4·7.

Another count seems worth recording in view of the influence of the A_1 A_2 B O groups on the reactions of the anti-Le^b serum : it shows no disturbance of proportion.

Mating Le(a +) × Le(a −).

	Children.			
	Le(a +). A_1 A_2 B O.		Le(a −). A_1 A_2 B O.	
	Compatible.	Incompatible.	Compatible.	Incompatible.
Mothers Le(a −) .	11	3	28	11
,, Le(a +) .	11	5	10	1

This can be reduced to the 2 × 2 table :

	Children A_1 A_2 B O.	
Mother : child.	Compatible.	Incompatible.
Lewis groups same . . .	39	16
,, ,, different . .	21	4

for which $\chi^2 = 1{\cdot}6$ for 1 degree of freedom, indicating that no disturbance has been detected. In this count compatible children are those whose ABO group

would permit them to give a transfusion to their mother with the qualification that an A_1 child has been scored as incompatible if the mother is A_2.

More than half of the 79 families described in this paper were also tested with anti-Le^b serum. Of the blood samples tested those belonging to group O, A_2, B or A_2 B were, with four exceptions, agglutinated either by anti-Le^a or by anti-Le^b, but not by both. The four exceptions, all from unrelated persons, gave unequivocally negative reactions with both antisera. More families are being tested in the hope of throwing further light on the genes involved in the Lewis groups.

SUMMARY.

The Lewis blood groups of 571 unrelated persons have been determined: 130 or 22·77 per cent were Le(a +). The Lewis blood groups of 79 families are given in detail, and on analysis the results are found to support strongly the contention of Andresen that adults whose red cells are Le(a +) are genetically homozygous.

The saliva of 172 members of these families has been tested for the presence of the A, B or H substances. The results entirely conform with the observation of Grubb that there is a very close, if not absolute, association between Le(a +) red cells and non-secretory saliva, and that the converse relationship, between Le(a −) red cells and secretory saliva is very close, though not absolute.

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